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(FILE 'HOME' ENTERED AT 18:22:03 ON 22 MAY 2003)

FILE 'MEDLINE, CAPLUS, BIOSIS, SCISEARCH' ENTERED AT 18:22:15 ON 22 MAY 2003

L1 2552 S FABRY(W)DISEASE OR DEFICIEN?(5A)ALPHA(W)GALACTOSIDASE(W)A
L2 93401 S GENE(W)THERAPY OR ENZYME(3A)REPLACEMENT(3A)THERAPY
L3 232 S L1(S)L2
L4 184 S L1(10A)L2
L5 168 S L1(6A)L2
L6 94 DUP REM L5 (74 DUPLICATES REMOVED)

=> d au ti so 51-94 l6

L6 ANSWER 51 OF 94 MEDLINE DUPLICATE 24
AU Pastores G M; Thadhani R
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SO LANCET, (2001 Aug 25) 358 (9282) 601-3.
Journal code: 2985213R. ISSN: 0140-6736.

L6 ANSWER 52 OF 94 MEDLINE
AU Desnick R J; Wasserstein M P
TI **Fabry disease**: clinical features and recent advances in **enzyme replacement therapy**.
SO ADVANCES IN NEPHROLOGY FROM THE NECKER HOSPITAL, (2001) 31 317-39. Ref: 73
Journal code: 0311622. ISSN: 0084-5957.

L6 ANSWER 53 OF 94 CAPLUS COPYRIGHT 2003 ACS
AU Fan, Jian-Qiang
TI Potential drug therapies for lysosomal storage disorders
SO Frontiers of Biotechnology & Pharmaceuticals (2001), 2, 275-291
CODEN: FBPRBL

L6 ANSWER 54 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Eng, C. M. (1); Cochat, P. (1); Wilcox, W. R. (1); Germain, D. P. (1); Waldek, S. (1); Caplan, L. (1); Heymans, H. (1); Desnick, R. J.
TI **Enzyme replacement therapy** for **Fabry disease**: Results of a multinational placebo-controlled phase 3 trial and open label extension study.
SO Genetics in Medicine, (May June, 2001) Vol. 3, No. 3, pp. 244. print.
Meeting Info.: Annual Clinical Genetics Meeting Miami, FL, USA March 01-04, 2001
ISSN: 1098-3600.

L6 ANSWER 55 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Schiffmann, R. (1); Kopp, J. B.; Moore, D. F. (1); Weibel, T. (1); Charria, G. (1); Brady, R. O. (1)
TI Efficacy and safety of prolonged **enzyme replacement therapy** for **Fabry disease**.
SO American Journal of Human Genetics, (October, 2001) Vol. 69, No. 4 Supplement, pp. 222. print.
Meeting Info.: 51st Annual Meeting of the American Society of Human Genetics San Diego, California, USA October 12-16, 2001
ISSN: 0002-9297.

L6 ANSWER 56 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AU Schiffmann R (Reprint); Kopp J B; Moore D F; Weibel T; Charria G; Brady R O
TI Efficacy and safety of prolonged **enzyme replacement therapy** for **Fabry disease**.

SO AMERICAN JOURNAL OF HUMAN GENETICS, (OCT 2001) Vol. 69, No. 4, Supp. [1], pp. 222-222. MA 234.
Publisher: UNIV CHICAGO PRESS, 1427 E 60TH ST, CHICAGO, IL 60637-2954 USA.
ISSN: 0002-9297.

L6 ANSWER 57 OF 94 CAPLUS COPYRIGHT 2003 ACS
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TI Fabry disease (.alpha.-galactosidase A deficiency): renal involvement and enzyme replacement therapy
SO Contributions to Nephrology (2001), 136(Rare Kidney Diseases), 174-192
CODEN: CNEPDD; ISSN: 0302-5144

L6 ANSWER 58 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Knecht, A. (1); Reznik-Wolf, H.; Serban, I.; Holtzman, E. J.
TI **Enzyme replacement therapy** for **Fabry disease**: A novel mutation in the alpha-galactosidase A gene and the initial results from the first patient to be treated in Israel.
SO Journal of Inherited Metabolic Disease, (2001) Vol. 24, No. Supplement 2, pp. 145-146. http://www.kluweronline.com/issn/0141-8955. print.
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ISSN: 0141-8955.

L6 ANSWER 59 OF 94 MEDLINE DUPLICATE 25
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SO JOURNAL OF INHERITED METABOLIC DISEASE, (2001) 24 Suppl 2 25-41; discussion 11-2. Ref: 105
Journal code: 7910918. ISSN: 0141-8955.

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AU Brady R O; Murray G J; Moore D F; Schiffmann R
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Journal code: 7910918. ISSN: 0141-8955.

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Journal code: 0370475. ISSN: 0002-9297.

L6 ANSWER 62 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AU Moore D F (Reprint); Scott L J C; Gladwin M T; Altarescu G; Kaneski C; Pease-Fye M; Ferri R; Brady R O; Herscovitch P; Schiffmann R
TI Regional cerebral hyperperfusion and nitric oxide pathway dysregulation in **Fabry disease** reversed by **enzyme replacement therapy**
SO ANNALS OF NEUROLOGY, (SEP 2001) Vol. 50, No. 3, Supp. [1], pp. S12-S12.
Publisher: WILEY-LISS, DIV JOHN WILEY & SONS INC, 605 THIRD AVE, NEW YORK, NY 10158-0012 USA.
ISSN: 0364-5134.

L6 ANSWER 63 OF 94 CAPLUS COPYRIGHT 2003 ACS
IN Yew, Nelson S.; Ziegler, Robin J.; Cheng, Seng H.
TI Compositions and methods for treating lysosomal storage disease
SO PCT Int. Appl., 54 pp.
CODEN: PIXXD2

L6 ANSWER 64 OF 94 CAPLUS COPYRIGHT 2003 ACS

IN Selden, Richard F.; Borowski, Marianne; Gillispie, Frances P.; Kinoshita, Carol M.; Treco, Douglas A.; Williams, Melanie D.
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SO U.S., 32 pp.
CODEN: USXXAM

L6 ANSWER 65 OF 94 MEDLINE DUPLICATE 28
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L6 ANSWER 67 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AU Moore D F (Reprint); Altarescu G; Herscovitch P; Schiffmann R
TI Increased cerebrovascular capacity following **enzyme replacement therapy** in **Fabry disease** demonstrated by positron emission tomography: A double-blind, randomized, placebo-controlled trial
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Publisher: LIPPINCOTT WILLIAMS & WILKINS, 530 WALNUT ST, PHILADELPHIA, PA 19106-3621.
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L6 ANSWER 68 OF 94 MEDLINE DUPLICATE 30
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SO PROTEIN EXPRESSION AND PURIFICATION, (2000 Nov) 20 (2) 228-36.
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L6 ANSWER 69 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Eng, C. (1); Phelps, R. (1); Kim, L. (1); Goldman, M. (1); Gordon, R. (1); Gass, A. (1); Winston, J. (1); Brodie, S. (1); Mehta, D. (1); Dikman, S. (1); Fallon, J. (1); Parsons, R. (1); Stacy, C. (1); Shinnar, M. (1); Rosenberg, M.; O'Callaghan, M.; Fitzpatrick, M.; Huertas, P.; Desnick, R. (1)
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SO Journal of Inherited Metabolic Disease, (July, 2000) Vol. 23, No. Supplement 1, pp. 222. print.
Meeting Info.: VIIIth International Conference on Inborn Errors of Metabolism England, Cambridge, UK September 13-17, 2000
ISSN: 0141-8955.

L6 ANSWER 70 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AU Schiffmann R (Reprint); Kopp J B; Austin H; Moore D F; Sabnis S; Weibel T; Balow J E; Brady R O
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ISSN: 0002-9297.

L6 ANSWER 71 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
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ISSN: 0002-9297.

L6 ANSWER 72 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Eng, C. M. (1); Cochat, P. (1); Wilcox, W. R. (1); Germain, D. P. (1); Lee, P. (1); Waldek, S. (1); Caplan, L. (1); Heymans, H. (1); Braakman, T.; Fitzpatrick, M. A.; Huertas, P.; O'Callaghan, M. W.; Richards, S.; Tandon, P. K.; Desnick, R.
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SO American Journal of Human Genetics, (October, 2000) Vol. 67, No. 4 Supplement 2, pp. 38. print.
Meeting Info.: 50th Annual Meeting of the American Society of Human Genetics Philadelphia, Pennsylvania, USA October 03-07, 2000 American Society of Human Genetics
. ISSN: 0002-9297.

L6 ANSWER 73 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Schiffmann, R. (1); Kopp, J. B.; Austin, H.; Moore, D. F. (1); Sabnis, S.; Weibel, T. (1); Balow, J. E.; Brady, R. O. (1)
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Meeting Info.: 50th Annual Meeting of the American Society of Human Genetics Philadelphia, Pennsylvania, USA October 03-07, 2000 American Society of Human Genetics
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L6 ANSWER 74 OF 94 SCISEARCH COPYRIGHT 2003 THOMSON ISI
AU Moore D (Reprint); Altarescu G; Ling G; Weibel T; Charria G; Schiffmann R
TI Increased transcranial Doppler parameters consistent with arterial recanalization following **enzyme replacement therapy** in **Fabry disease**
SO NEUROLOGY, (27 JUN 2000) Vol. 54, No. 12, pp. 2-2.
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Journal code: 9808008. ISSN: 1434-5161.

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SO HUMAN GENE THERAPY, (1999 Dec 10) 10 (18) 2881-9.
Journal code: 9008950. ISSN: 1043-0342.

L6 ANSWER 77 OF 94 CAPLUS COPYRIGHT 2003 ACS
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TI Circulating .alpha.-galactosidase a derived from transduced bone marrow cells: relevance for corrective gene transfer for Fabry disease
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L6 ANSWER 78 OF 94 CAPLUS COPYRIGHT 2003 ACS
AU Ziegler, Robin J.; Yew, Nelson S.; Li, Chester; Cherry, Maribeth; Berthelette, Patricia; Romanczuk, Helen; Ioannou, Yiannis A.; Zeidner, Kenneth M.; Desnick, Robert J.; Cheng, Seng H.
TI Correction of enzymatic and lysosomal storage defects in Fabry mice by adenovirus-mediated gene transfer
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CODEN: EXHMA6; ISSN: 0301-472X

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Meeting Info.: 49th Annual Meeting of the American Society of Human Genetics San Francisco, California, USA October 19-23, 1999 The American Society of Human Genetics
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SO (1998) 230 pp. Avail.: UMI, Order No. DA9908386
From: Diss. Abstr. Int., B 1999, 59(9), 4613

L6 ANSWER 82 OF 94 CAPLUS COPYRIGHT 2003 ACS
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TI Gene and enzyme replacement therapy for .alpha.-galactosidase A deficiency
SO PCT Int. Appl., 78 pp.
CODEN: PIXXD2

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SO AMERICAN JOURNAL OF HUMAN GENETICS, (OCT 1997) Vol. 61, No. 4, Supp. [S], pp. 2108-2108.
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ISSN: 0002-9297.

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SO LEUKEMIA, (1997 Apr) 11 Suppl 3 552-6.
Journal code: 8704895. ISSN: 0887-6924.

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Meeting Info.: 47th Annual Meeting of the American Society of Human Genetics Baltimore, Maryland, USA October 28-November 1, 1997
ISSN: 0002-9297.

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CODEN: PNASA6; ISSN: 0027-8424

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Meeting Info.: Joint Meeting of the American Society for Biochemistry and Molecular Biology, the American Society for Investigative Pathology and the American Association of Immunologists New Orleans, Louisiana, USA June 2-6, 1996
ISSN: 0892-6638.

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TI **GENE-THERAPY** FOR **FABRY DISEASE** - EXPRESSION, SECRETION AND UPTAKE OF ALPHA-GALACTOSIDASE-A (ALPHA-GAL-A) DRIVEN BY A HIGH-TITER RECOMBINANT RETROVIRAL
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ISSN: 0892-6638.

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AU Ioannou, Y. A. (1); Zeidner, K. M. (1); Friedman, B.; Desnick, R. J. (1)
TI **Fabry disease**: **Enzyme replacement therapy** in alpha-galactosidase A deficient mice.
SO American Journal of Human Genetics, (1996) Vol. 59, No. 4 SUPPL., pp. A15.
Meeting Info.: 46th Annual Meeting of the American Society of Human Genetics San Francisco, California, USA October 29-November 2, 1996

ISSN: 0002-9297.

L6 ANSWER 91 OF 94 MEDLINE DUPLICATE 33
AU Sugimoto Y; Aksentijevich I; Murray G J; Brady R O; Pastan I; Gottesman M M
TI Retroviral coexpression of a multidrug resistance gene (MDR1) and human alpha-galactosidase A for **gene therapy** of **Fabry disease**.
SO HUMAN GENE THERAPY, (1995 Jul) 6 (7) 905-15.
Journal code: 9008950. ISSN: 1043-0342.

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TI EXPRESSION OF THE HUMAN ALPHA GALACTOSIDASE A IN ESCHERICHIA-COLI K-12.
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CODEN: GENED6. ISSN: 0378-1119.

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AU SVENNERHOLM L
TI INHERITED LYSOSOMAL DISEASE PATHOGENESIS DIAGNOSIS AND THERAPY.
SO 18TH NORDIC CONGRESS IN CLINICAL CHEMISTRY, REYKJAVIK, ICELAND, JUNE 23-26, 1981. SCAND J CLIN LAB INVEST SUPPL. (1981 (RECD 1982)) 41 (158), 6.
CODEN: SCLSAH. ISSN: 0085-591X.

L6 ANSWER 94 OF 94 CAPLUS COPYRIGHT 2003 ACS
AU Bishop, David F.; Dean, Kenneth J.; Sweeley, Charles C.; Desnick, Robert J.
TI Purification and characterization of human .alpha.-galactosidase isoenzymes: comparison of tissue and plasma forms and evaluation of purification methods
SO Birth Defects, Original Article Series (1980), 16(1, Enzyme Ther. Genet. Dis. 2), 17-32
CODEN: BTHDAK; ISSN: 0547-6844

=> d bib ab 82 16

L6 ANSWER 82 OF 94 CAPLUS COPYRIGHT 2003 ACS
AN 1998:183999 CAPLUS
DN 128:239483
TI Gene and enzyme replacement therapy for .alpha.-galactosidase A deficiency
IN Selden, Richard F.; Borowski, Marianne; Gillespie, Frances P.; Kinoshita, Carol M.; Treco, Douglas A.; Williams, Melanie D.
PA Transkaryotic Therapies, Inc., USA; Selden, Richard F.; Borowski, Marianne; Gillespie, Frances P.; Kinoshita, Carol M.; Treco, Douglas A.; Williams, Melanie D.
SO PCT Int. Appl., 78 pp.
CODEN: PIXXD2
DT Patent
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9811206	A2	19980319	WO 1997-US16603	19970912
	WO 9811206	A3	19980813		

W: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM
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	AU 9744244	A1	19980402	AU 1997-44244	19970912
	EP 935651	A2	19990818	EP 1997-942567	19970912
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
	CN 1230220	A	19990929	CN 1997-197909	19970912
	NZ 334721	A	20010126	NZ 1997-334721	19970912
	JP 2001504324	T2	20010403	JP 1998-514004	19970912
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	NZ 506214	A	20021126	NZ 1997-506214	19970912
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	NO 9901225	A	19990510	NO 1999-1225	19990312
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	US 2003077806	A1	20030424	US 2002-165060	20020607
PRAI	US 1996-712614	A	19960913		
	US 1996-26041P	P	19960913		
	NZ 1997-334721	A1	19970912		
	US 1997-928881	A2	19970912		
	WO 1997-US16603	W	19970912		
	US 1999-266014	A3	19990311		

AB A therapeutic method is provided whereby an individual suspected of having an .alpha.-galactosidase A (.alpha.-gal A) deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human .alpha.-gal A, or (2) purified human .alpha.-gal A obtained from cultured, genetically modified human cells. Expressing a DNA encoding human .alpha.-gal A in cultured human cells produces a polypeptide that is glycosylated appropriately, so that it is not only enzymically active and capable of acting on the glycosphingolipid substrate which accumulates in Fabry disease, but is also efficiently internalized by cells via cell surface receptors which target it exactly to where it is needed in this disease. Two expression plasmids, pXAG-16 and pXAG-28, were constructed. These plasmids contain human .alpha.-gal A cDNA encoding the 398 amino acids of the .alpha.-gal A enzyme (without its signal peptide); the human growth hormone (hGH) signal peptide genomic DNA sequence, which is interrupted by the first intron of the hGH gene; and the 3'-untranslated sequence (UTS) of the hGH gene, which contains a signal for polyadenylation. Plasmid pXAG-16 has the human cytomegalovirus immediate-early promoter and first intron (flanked by noncoding exon sequences), whereas pXAG-28 is driven by the collagen I.alpha.2 promoter and also contains the .beta.-actin gene's 5'-UTS, which contains the first intron of the .beta.-actin gene. Expression by fibroblasts stably transfected with pXAG-16 or pXAG-28, using the hGH signal peptide, was substantially higher than that in transfected fibroblasts using the homologous .alpha.-gal A signal peptide. Recombinant .alpha.-gal A could be purified by Butyl-Sepharose hydrophobic interaction chromatog., heparin-Sepharose chromatog., hydroxylapatite chromatog., Q Sepharose HP anion-exchange chromatog., and Superdex-200 gel filtration chromatog. Purified .alpha.-Gal A activity was stable over a 3-mo period when the pH of the formulation was <6.5.

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L6 ANSWER 91 OF 94 MEDLINE DUPLICATE 33
AU Sugimoto Y; Aksentijevich I; Murray G J; Brady R O; Pastan I; Gottesman M M
TI Retroviral coexpression of a multidrug resistance gene (MDR1) and human alpha-galactosidase A for gene therapy of Fabry disease.
SO HUMAN GENE THERAPY, (1995 Jul) 6 (7) 905-15.

L6 ANSWER 90 OF 94 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AU Ioannou, Y. A. (1); Zeidner, K. M. (1); Friedman, B.; Desnick, R. J. (1)
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HUMAN GENE THERAPY 6:905–915 (July 1995)
Mary Ann Liebert, Inc.

Retroviral Coexpression of a Multidrug Resistance Gene (*MDR*1) and Human α-Galactosidase A for Gene Therapy of Fabry Disease

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ABSTRACT

Human α-galactosidase A (α-Gal A; EC. 3.2.1.22) is a lysosomal exoglycosidase encoded by a gene on Xq22. Deficiencies of this enzyme result in Fabry disease, an X-chromosome-linked recessive disorder that leads to premature death in affected males. For treatment of genetic diseases, we have developed a retroviral vector system, pSXLC/pHa, that enables coexpression of drug-selectable markers with a second nonselectable gene as part of a bicistronic message using the promoter from the Harvey murine sarcoma virus and an internal ribosomal entry site (IRES) from encephalomyocarditis virus. Retroviral vectors based on this system that carry the human α-Gal A cDNA either upstream (pHa-αGal-IRES-MDR) or downstream (pHa-MDR-IRES-αGal) from the IRES relative to the drug-selectable *MDR*1 (P-glycoprotein) cDNA were constructed. Each of eight independent vincristine-resistant, pHa-αGal-IRES-MDR-transfected clones and all four vincristine-resistant, pHa-αGal-IRES-MDR retrovirus-transduced clones showed significantly higher activity of α-Gal A than the parental cells. More than 50% of the vincristine-resistant, pHa-MDR-IRES-αGal-transfected clones and all four vincristine-resistant, pHa-MDR-IRES-αGal retrovirus-transduced clones showed significantly higher activity of α-Gal A than the parental cells. In these bicistronic vectors, the cDNA whose translation was cap-dependent (upstream) was expressed at higher levels than when the same cDNA was translated in an IRES-dependent manner (downstream). These vectors may prove useful in the gene therapy of Fabry disease.

OVERVIEW SUMMARY

The use of the human multidrug-resistant gene (*MDR*1) as a selectable marker for retroviral vectors should allow for selection of transduced cells coexpressing the therapeutic gene. Sugimoto *et al.* constructed a retroviral vector system in which the second gene is expressed under control of an internal ribosome entry site and used this system to coexpress the drug-selectable *MDR*1 cDNA and α-galactosidase A cDNA for gene therapy of Fabry disease. This work demonstrates the efficient coexpression of the two transduced genes after retrovirus-mediated gene transfer.

INTRODUCTION

GENE THERAPY MAY BE USEFUL for the treatment of cancer and many inherited diseases (Miller, 1992; Mulligan, 1993). In many cases, foreign genes have been introduced into mammalian cells using retrovirus-mediated gene transfer, but the efficiency of transfer and the stability of the transferred genes may be limited (Dzierzak *et al.*, 1988; Bodine *et al.*, 1989; Bodine *et al.*, 1990; Fletcher *et al.*, 1991; Olsen *et al.*, 1993). The use of a dominant drug-selectable marker *in vitro* and *in vivo* should allow for the selection and enrichment of cells expressing the transduced gene.

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The study of the mechanisms of resistance of cancer cells to anticancer drugs has led to the identification of some of the genes and molecules that confer drug resistance. Among them, cell lines showing resistance to multiple drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and actinomycin D have been studied intensively, and the gene responsible for this form of multidrug resistance in human cells, termed *MDR*1, has been identified (Chen *et al.*, 1986; Ueda *et al.*, 1987a). The *MDR*1 gene encodes the 170,000-dalton plasma membrane P-glycoprotein. P-glycoprotein acts as an ATP-dependent efflux pump for various structurally unrelated natural product antitumor agents (for review, see Gottesman and Pastan, 1993). The *MDR*1 cDNA has been shown to confer multidrug resistance when expressed in drug-sensitive cells (Ueda *et al.*, 1987b; Pastan *et al.*, 1988). Retrovirus-mediated expression of the *MDR*1 cDNA has been shown to confer multidrug resistance *in vivo* when the *MDR*1-carrying vector is introduced into the bone marrow cells of mice (Podda *et al.*, 1992; Sorrentino *et al.*, 1992). Studies using *MDR*1 transgenic mice suggest that the expression of the *MDR*1 cDNA in bone marrow cells does not affect the normal function of the bone marrow cells (Galski *et al.*, 1989; Mickisch *et al.*, 1991). Therefore, in principle, the *MDR*1 gene could be used as a dominant selectable marker *in vivo* that allows for the selection and enrichment of cells coexpressing a second transduced gene.

We have developed a retroviral vector system, pSXLC/pHa, that utilizes a putative internal ribosome entry site (IRES) isolated from encephalomyocarditis virus (Kaufman *et al.*, 1991; Morgan *et al.*, 1992; Dirks *et al.*, 1993). In this construct, a single mRNA is transcribed under control of an upstream promoter, and two gene products are translated independently from a bicistronic mRNA. One of these gene products is translated in a cap-dependent manner and the other is translated under control of the IRES. The *MDR*1 gene is efficiently expressed in this system under the control of the IRES (Sugimoto *et al.*, 1994).

In the current work, we used the pSXLC/pHa system to coexpress a human α-galactosidase A (α-GalA; EC.3.2.1.22) cDNA with an *MDR*1 cDNA as a potential gene therapy of Fabry disease. Fabry disease results from mutations in the α-Gal A gene that cause progressive glycosphingolipid deposition leading to early demise from renal, cardiac, or cerebrovascular disease (for review, see Desnick and Bishop, 1989). No effective treatment exists. Enzyme replacement therapy may be relatively ineffective for the treatment of Fabry disease because the half-life of injected α-Gal A, at least in the circulation, is very short (Brady *et al.*, 1973). Coexpression of the *MDR*1 gene with α-Gal A may be effective for treatment of Fabry disease for the following reasons: (i) Since deficiencies in the single-chain enzyme α-Gal A cause the disease, expression of a normal cDNA for α-Gal A should cure the disease; and (ii) the disease is apparently due to the ineffectiveness of macrophages to digest glycosphingolipids. Therefore, correction of the enzyme deficiency in macrophages and other cells by the introduction of cDNA for the deficient enzyme into bone marrow cells may result in the catabolism of ceramide trihexoside arising from erythrocytorrhexis.

In this report, we describe the activity of two retroviral constructs. One vector is pHa-αGal-IRES-MDR which has an α-Gal A cDNA upstream from the IRES and the *MDR*1 cDNA under control of the IRES, and the other is pHa-MDR-IRES-αGal which has the *MDR*1 cDNA upstream from the IRES and the α-Gal A cDNA under control of the IRES. These vectors confer multidrug resistance with high expression of human P-glycoprotein. Most of the drug-resistant cells showed higher activity of α-Gal A than the parental cells, with levels dependent on whether the *MDR*1 cDNA was upstream or downstream from the IRES. High titers of amphotropic retrovirus capable of transducing cultured cells were obtained with these vectors. These results suggest that the *MDR*1 gene may be useful as a dominant selectable marker in gene therapy of Fabry disease.

MATERIALS AND METHODS

Cell culture and assay of drug sensitivity

The ecotropic retrovirus packaging cell line Ψ-cre, the amphotropic retrovirus packaging cell line Ψ-crip (Danos and Mulligan, 1988), and the mouse fibroblast cell line NIH-3T3 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum. The amphotropic retrovirus packaging cell line PA317 (Miller and Buttimore, 1986) was grown in medium supplemented with 10% fetal bovine serum (FBS). The sensitivity of the cells to vincristine was evaluated by inhibition of cell growth after incubation at 37°C for 6 days in the presence of various concentrations of the drug as described previously (Sugimoto and Tsuruo, 1987). Cell numbers were determined in a Coulter counter, and the concentration of drug that inhibits cell growth by 50% (IC_{50}) was determined. Data are expressed as means of triplicate determinations.

Isolation of the cDNA for human α-Gal A

The human α-Gal A cDNA was isolated by reverse transcription-polymerase chain reaction (RT-PCR) according to the published sequence of α-Gal A (Bishop *et al.*, 1986, 1988; Tsuji *et al.*, 1987). We first used three sets of PCR primers to isolate three pieces of the cDNA from human KB-3-1 cells. The sequences of PCR primers are as follows: sense primer 1 (−24 to −1) 5′-TTATGCTGTCCGGTCACCGTGACA-3′ and antisense primer 2 (531–508) 5′-CAAACTGTCACAGTAACAACCATC-3′; sense primer 3 (472–495) 5′-ACCTTTGCTGACTGGGGAGTAGAT-3′ and antisense primer 4 (853–830) 5′-CCATCTGAGTTACTTGCTGATTCC-3′; sense primer 5 (804–827) 5′-AGTGATTGGCAACTTTGGCCTCAG-3′ and antisense primer 6 (1,254–1,231) 5′-TTCTAGCTGAAGCAAAACAGTGCC-3′. After RT-PCR, each cDNA was subcloned into *Sma* I-digested pGEM7Z. The α-Gal A cDNA has two potential polyadenylation signals (^{1,255}AATACA and ^{1,272}ATTAAA). To inactivate these two polyadenylation sites, we made a synthetic adaptor (60-mer/67-mer) which has the sequence of α-Gal A cDNA from 1,231 (*Alw* NI site) to 1,288 (3 bases after the termination codon) and introduces nonsense mutations in each polyadenylation signal (^{1,255}AA<u>C</u>ACA and ^{1,272}<u>T</u>TTAAA) as well as a 3′-*Xho* I site at the 3′ end of the cDNA. The adaptor was ligated to the *Alw* NI-digested 3′-fragment (804–1,231) of the α-Gal A cDNA. The three cDNA fragments were ligated together after digestion with *Pvu* II (nucleotide 492) and *Bgl* II (nucleotide 825). The resulting plasmid with the full-length α-Gal A cDNA was termed pGEM7/αGal.

The nucleotide sequence of the α-Gal A cDNA was confirmed using an automatic sequencer (373A, Applied Biosystems, Foster City, CA).

Construction of vectors

The basic structure of the pSXLC/pHa retrovirus system has been described (Sugimoto *et al.*, 1994). The plasmid pSXLC-MDR has the entire open reading frame of the *MDR*1 cDNA downstream from the IRES sequence and five unique sites (*Sac* II, *Bam* HI, *Sac* I, *Xba* I, and *Sal* I) for the cloning of another gene upstream from the IRES. The construct pHa-αGal-IRES-MDR has the α-Gal A cDNA upstream from the IRES. To make this construct, we first destroyed the 3′ *Xho* I site of pGEM7/αGal, then cloned the *Bam* HI, *Xba* I insert of the α-Gal A cDNA into the *Bam* HI, *Xba* I-digested pSXLC-MDR (pSXLC-αGal-MDR). The pSXLC-αGal-MDR insert was isolated after *Sac* II, *Xho* I-digestion and transferred into the pHa retroviral vector.

A second construct was pHa-MDR-IRES-αGal, which has the α-Gal A cDNA downstream from the IRES. To insert cDNA downstream from the IRES sequence of pSXLC, we used the ATG codon within the single *Nco* I site (CCATGG) at the 3′ end of the IRES sequence. The second C nucleotide may be important for efficient translation (Kozak, 1984). To maintain the CCATGG sequence, we used two synthetic adaptors from nucleotide −1 (newly created *Nco* I end) to +35 (unique *Bss* HII site) of α-Gal A cDNA. The original amino acid sequence of α-Gal A at its amino terminus is Met-Gln-Leu-Arg. The 5′ nucleotide sequence of the 36-mer adaptor is CATGGCCCTGAGG, which changes the second amino acid from Gln to Ala (Met-Ala-Leu-Arg). The 5′ sequence of the 42-mer adaptor is CATGGCCATGCAGCTGAGG, which adds Met-Ala at the amino terminus of the signal peptide (*Met-Ala*-Met-Gln-Leu-Arg). These adaptors were ligated with the *Bss* HII–*Xho* I fragment of α-Gal A cDNA and the full-length α-Gal A cDNAs were subcloned into pSXLC. These plasmids, pSXLC-αGal(1) with the 35-mer adaptor and pSXLC-αGal(2) with the 41-mer adaptor, have four unique sites (*Sac* II, *Bam* HI, *Xba* I, and *Sal* I) for cloning of another gene. Next we subcloned the *Sac* II, *Xba* I-digested *MDR* 1 cDNA between the *Sac* II and *Xba* I sites of pSXLC-αGal(1) and pSXLC-αGal(2). The inserts of these plasmids were isolated after *Sac* II, *Xho* I digestion and transferred into the pHa retroviral vector. These retrovirus constructs were termed pHa-MDR-IRES-αGal(1) and pHa-MDR-IRES-αGal(2). The amino-terminal sequences of α-Gal A cDNA in these retroviral constructs are summarized in Table 1.

TABLE 1. AMINO-TERMINAL STRUCTURES OF α-Gal A

Construct	*Sequence*
Wild-type α-Gal A	ATG CAG CTG AGG Met-Gln-Leu-Arg
pHa-αGal-IRES-MDR	ATG CAG CTG AGG Met-Gln-Leu-Arg
pHa-MDR-IRES-αGal(1)	ATG GCC CTG AGG Met-Ala-Leu-Arg
pHa-MDR-IRES-αGal(2)	ATG GCC ATG CAG CTG AGG Met-Ala-Met-Gln-Leu-Arg

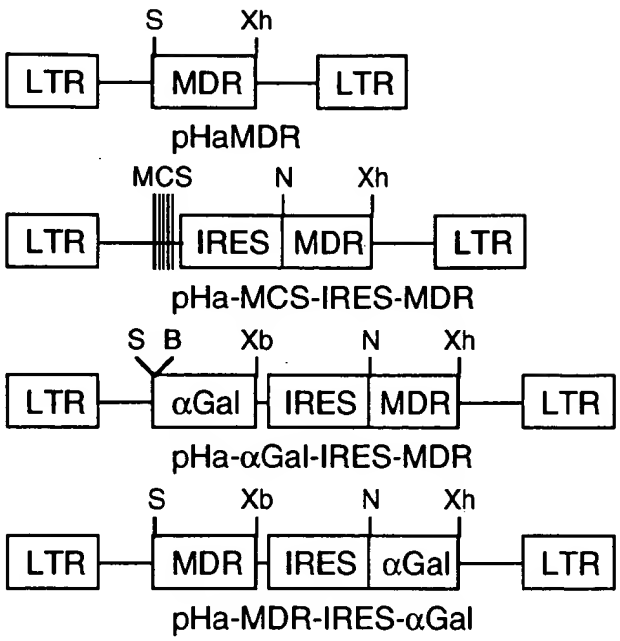

FIG. 1. Structure of pHaMDR, pHa-MCS-IRES-MDR, pHa-αGal-IRES-MDR, and pHa-MDR-IRES-αGal retroviruses. Drawing is not to scale. LTR, Long terminal repeat of Harvey murine sarcoma virus; MDR, human multidrug resistance gene *MDR*1 cDNA; IRES, internal ribosome entry site; αGal, human α-Gal A cDNA; S, *Sac* II; Xh, *Xho* I; MCS, multicloning site (*Sac* II-*Bam* HI-*Bgl* II-*Sac* I-*Xba* I-*Sal* I); N, *Nco* I; B, *Bam* HI; Xb, *Xba* I.

Two *MDR*1-carrying retroviral constructs were used as control vectors. The pHaMDR construct carries the wild-type *MDR*1 cDNA in the pHa retrovirus vector (Pastan *et al.*, 1988; Kioka *et al.*, 1989). The pHa-MCS-IRES-MDR construct carries the *MDR*1 cDNA under the control of the IRES (Sugimoto *et al.*, 1994). The schematic structures of the retroviral constructs are presented in Fig. 1.

DNA transfection

Transfection was carried out using the high-efficiency calcium phosphate coprecipitation method (Chen and Okayama, 1987). Ψ-cre cells were plated at 5×10^5 cells per 100-mm dish on day 1 and transfected with 20 μg of the expression plasmid DNA on day 2. Cells were exposed to the DNA precipitate until day 3, when the medium was aspirated and fresh medium was added. On day 4, the cells were split at 1:10 or 1:100. The cells were selected with 25 ng/ml vincristine.

Retrovirus transduction

Retrovirus-producing cells were plated on day 1 at 2×10^6 cells per 100-mm dish. On day 2, the medium of the packaging cell culture was changed, and the recipient cells were plated at 3×10^4 cells per 100-mm dish in medium containing 2 μg/ml Polybrene (Aldrich, Milwaukee, WI). On day 3, the retrovirus-containing supernatant was collected, passed through a 0.45-μm-pore filter to remove cells and debris, and added to each dish of recipient cells. On day 5, medium was removed and

fresh medium containing vincristine (25 ng/ml for Ψ-crip and NIH-3T3, 30 ng/ml for PA317) was added. On days 11–13, the medium was removed and the colonies were stained with 0.5% methylene blue dissolved in 50% methanol.

FACS analysis

To examine the expression of human P-glycoprotein on the cell surface of mouse transfectants, FACS analysis was done with a human P-glycoprotein-specific monoclonal antibody MRK16 (Hamada and Tsuruo, 1986; Ishida *et al.*, 1989). Cells (10^6) harvested after trypsinization were washed and incubated with MRK16 (5 $\mu g/10^6$ cells), washed twice, and incubated with fluorescein-conjugated goat anti-mouse IgG (1:10 diluted, Jaxon Immunoresearch Lab., West Grove, PA). The cells were washed twice and the fluorescence staining level was analyzed using a FACSort (Beckton-Dickinson FACS System, San Jose, CA). Mean channel number of the fluorogram was used to compare the expression levels of human P-glycoprotein among transfectant and transductant clones.

Enzyme activity assay

Cell pellets in microtubes were resuspended in 10 volumes of extraction buffer containing 50 m*M* phosphate buffer pH 6.0, 0.2% Triton X-100, and 20 μg/ml aprotinin (Sigma, St. Louis, MO) and sonicated on ice for 90 sec. Lysates were centrifuged at 14,000 rpm for 30 sec. The resulting supernatants were used as cell extracts. Protein concentrations were determined by the Protein Assay Kit (Bio-Rad, Hercules, CA). The α-Gal A activity in the cell lysate was determined using 3.5 m*M* 4-methylumbelliferyl-α-D-galactopyranoside as a substrate in the presence of 100 m*M* *N*-acetylgalactosamine (Ioannou *et al.*, 1992). Data (percent of activity in parental cells) are expressed as means of four determinations.

Western blot analysis

Cell extracts prepared as described above were separated on SDS-polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes. For the detection of P-glycoprotein, the blot was incubated with 0.5 μg/ml of C219 monoclonal antibody (Kartner *et al.*, 1985), washed, and incubated with peroxidase-conjugated goat anti-mouse IgG (1:1000 diluted, Sigma). For the detection of α-Gal A, the blot was incubated with 1:125-diluted rabbit anti-α-Gal A antibody (Kusiak *et al.*, 1978), washed, and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:4,000 diluted, Sigma). The peroxidase on the blot was visualized using the ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL).

RESULTS

DNA transfection

The retroviral expression constructs, pHaMDR, pHa-MCS-IRES-MDR, pHa-αGal-IRES-MDR, pHa-MDR-IRES-αGal(1), and pHa-MDR-IRES-αGal(2) were transfected into Ψ-cre, a murine retrovirus-packaging cell line as described in Materials and Methods. After 10 days, approximately 30 vincristine-resistant colonies per dish were observed in pHa-αGal-IRES-MDR-transfected cells when transfected cells were split 1:100 on day 4 (transfection efficiency was 1.1×10^{-3}). This transfection efficiency was three-fold less than that of pHa-MCS-IRES-MDR (3.2×10^{-3}), a control *MDR*1 vector whose *MDR*1 gene is also controlled by the IRES, and seven-fold less than that of pHaMDR (7.5×10^{-3}). In contrast, the two pHa-MDR-IRES-αGal constructs [αGal(1) and αGal(2)] conferred multidrug resistance similar to pHaMDR (transfection efficiencies were 6.4×10^{-3} and 6.1×10^{-3}, respectively).

Retrovirus production

To test the ability of pHa-αGal-IRES-MDR and pHa-MDR-IRES-αGal to be packaged as retrovirus, culture supernatants of pooled vincristine-resistant Ψ-cre (ecotropic packaging line) transfectants were added to the culture of amphotropic retrovirus-packaging lines Ψ-crip or PA317. After retrovirus transduction and subsequent vincristine selection, many drug-resistant colonies were obtained, indicating that retrovirus carrying the *MDR*1 gene existed in the supernatants. The retrovirus titer produced by mixed populations of Ψ-cre cells transfected with pHa-αGal-IRES-MDR was 1.1×10^3/ml when PA317 cells were used as recipient cells (Table 2). This titer was five-fold less than the titer of pHaMDR (titer 5.8×10^3/ml). On the other hand, retrovirus titers produced by mixed populations of Ψ-cre cells transfected with pHaMDR-IRES-αGal were 4×10^3/ml (Table 2).

The retrovirus titers of PA317 or Ψ-crip cells transduced with pHa-αGal-IRES-MDR or pHa-MDR-IRES-αGal (mixed populations) were examined using NIH-3T3 cells as recipient cells (Table 2). PA317 cells consistently produced somewhat higher titers of retrovirus than Ψ-crip cells. Retrovirus titers of

TABLE 2. TITERS OF THE *MDR*1 RETROVIRUSES

Vector	*Retrovirus titer*[a]		
	Ψ-cre	*Ψ-crip*	*PA317*
pHaMDR	$5.8 \pm 0.3 \times 10^3$	$2.8 \pm 0.5 \times 10^4$	$8.5 \pm 1.0 \times 10^4$
pHa-MCS-IRES-MDR	$3.1 \pm 0.1 \times 10^3$	$1.2 \pm 0.2 \times 10^4$	$2.9 \pm 0.4 \times 10^4$
pHa-αGal-IRES-MDR	$1.1 \pm 0.1 \times 10^3$	$8.0 \pm 1.2 \times 10^3$	$1.3 \pm 0.0 \times 10^4$
pHa-MDR-IRES-αGal(1)	$3.8 \pm 0.2 \times 10^3$	$2.9 \pm 0.2 \times 10^4$	$6.1 \pm 0.2 \times 10^4$
pHa-MDR-IRES-αGal(2)	$4.5 \pm 0.3 \times 10^3$	$2.5 \pm 0.0 \times 10^4$	$5.3 \pm 0.2 \times 10^4$

[a]Calculated number of vincristine-resistant colonies when NIH-3T3 cells were transduced with 1 ml of supernatant from a mixed population of retrovirus-producing cells and selected with 25 ng/ml vincristine. Standard deviations are shown.

pHa-αGal-IRES-MDR were 4- to 10-fold less than those of pHaMDR. Retrovirus titers of pHa-MDR-IRES-αGal were slightly less than those of pHaMDR. To obtain retrovirus-producing cells with higher titers, we isolated single PA317 or Ψ-crip clones transduced with pHa-αGal-IRES-MDR or pHa-MDR-IRES-αGal retrovirus. The highest titer of pHa-αGal-IRES-MDR retrovirus we obtained from 13 clones of PA317 was 3.0×10^4/ml, and the highest titers of pHa-MDR-IRES-αGal(1) and pHa-MDR-IRES-αGal(2) retrovirus from eight clones each of PA317 were 8.4×10^4/ml and 1.2×10^5/ml, respectively. We could not get higher titers of retrovirus than these from Ψ-crip clones.

Expression of human P-glycoprotein

Expression of P-glycoprotein on the cell surface of NIH-3T3 populations transduced with the vectors was examined by FACS with MRK16 antibody (Fig. 2). The fluorescence peaks of the

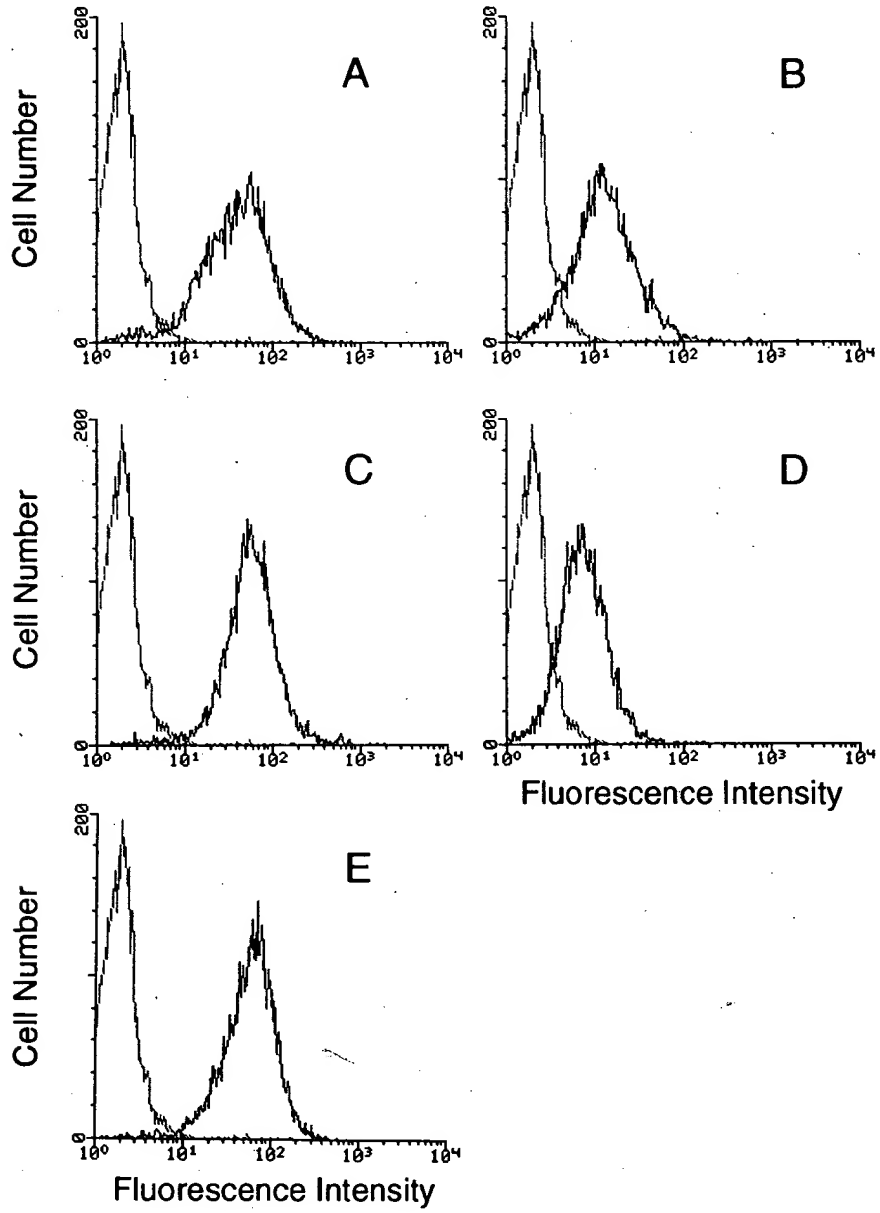

FIG. 2. FACS analysis of mixed populations of NIH-3T3 cells transduced with pHaMDR, pHa-MCS-IRES-MDR, pHa-αGal-IRES-MDR, and pHa-MDR-IRES-αGal. Cells (10^6) were harvested after trypsinization, stained with the anti-P-glycoprotein monoclonal antibody MRK16, washed, and stained with fluorescein-conjugated anti-mouse IgG. The fluorescence was analyzed by FACS. A. NIH-3T3 cells (left) and the pHaMDR-transduced NIH-3T3 population selected with vincristine (right). B. NIH-3T3 cells (left) and the pHa-MCS-IRES-MDR-transduced NIH-3T3 population selected with vincristine (right). C. NIH-3T3 cells (left) and the pHa-MDR-IRES-αGal(1)-transduced NIH-3T3 population selected with vincristine (right). D. NIH-3T3 cells (left) and the pHa-αGal-IRES-MDR-transduced NIH-3T3 population selected with vincristine (right). E. NIH-3T3 cells (left) and the pHa-MDR-IRES-αGal(2)-transduced NIH-3T3 population selected with vincristine (right).

transductants suggest that all the drug-selected cells express human P-glycoprotein. Mixed populations of the pHa-MDR-IRES-αGal(1) or pHa-MDR-IRES-αGal(2) transductants selected with vincristine showed levels of P-glycoprotein expression similar to pHaMDR transductants (Fig. 2). The pHa-αGal-IRES-MDR transductants showed slightly lower expression of P-glycoprotein than those with pHa-MCS-IRES-MDR, and much lower expression than those with pHaMDR (Fig. 2).

Coexpression of α-Gal A in drug-resistant transfectants

To determine whether the *MDR*1 and α-Gal A genes are co-expressed in clonal cell lines, we randomly isolated Ψ-cre transfectant clones and determined the activity of α-Gal A (Table 3). To examine the possible correlation between the expression of α-Gal A and vincristine resistance (P-glycoprotein expression), sensitivity to vincristine and expression of human P-glycoprotein were also determined in each clone (Table 3). Every one of the eight pHa-αGal-IRES-MDR-transfectant clones (ΨGM1-ΨGM8) possessed α-Gal A activity at least three-fold as high as the parental cells or the *MDR*1 transfectants. The pHa-αGal-IRES-MDR transfectants showed 5- to 10-fold higher resistance to vincristine than the parental cells. The cell-surface expression of human P-glycoprotein was confirmed by FACS using MRK16. The drug resistance of the pHa-αGal-IRES-MDR transfectants was lower than that observed in pHaMDR transfectants. However, this resistance is high enough to select transfected cells with drugs at doses toxic for the parental cells. On the other hand, only 8/12 pHa-MDR-IRES-αGal-transfectant clones (ΨMG11-ΨMG13 and ΨMG21-ΨMG25) showed increases in α-Gal A activity. The clones from ΨGM11 to ΨGM15 are pHa-MDR-IRES-αGal(1) transfectants, and the clones from ΨGM21 to ΨGM27 are pHa-MDR-IRES-αGal(2) transfectants. When the α-Gal A activity is compared with the vincristine resistance of each clone, the IC_{50} values for vincristine of the eight clones with the highest α-Gal A activity were higher than 54 ng/ml. The highest IC_{50} value for vincristine of the other four clones was 40 ng/ml.

TABLE 3. α-GAL A ACTIVITY, VINCRISTINE SENSITIVITY, AND P-GLYCOPROTEIN EXPRESSION IN Ψ-cre TRANSFECTANT CLONES

	α-Gal A activity (% of control)[a]	*IC_{50} to vincristine (ng/ml)*[a]	*P-glycoprotein expression*[b]
Ψ-cre	100 ± 7	2.8 ± 0.3	4
pHaMDR			
ΨM1	88 ± 5	121 ± 15	257
ΨM2	116 ± 13	88 ± 4	101
ΨM3	92 ± 4	55 ± 1	73
ΨM4	87 ± 6	51 ± 3	68
pHa-MCS-IRES-MDR			
ΨIM1	111 ± 18	70 ± 3	44
ΨIM2	97 ± 3	63 ± 4	nd
ΨIm3	95 ± 10	50 ± 1	nd[c]
pHa-αGal-IRES-MDR			
ΨGM1	378 ± 9	29 ± 1	nd
ΨGM2	351 ± 13	26 ± 0	nd
ΨGM3	324 ± 28	22 ± 3	16
ΨGM4	308 ± 6	22 ± 1	nd
ΨGM5	295 ± 12	21 ± 1	17
ΨGM6	330 ± 16	19 ± 2	13
ΨGM7	319 ± 15	17 ± 0	nd
ΨGM8	352 ± 48	14 ± 2	14
pHa-MDR-IRES-αGal(1)			
ΨMG11	314 ± 22	88 ± 3	98
ΨMG12	185 ± 3	66 ± 6	nd
ΨMG13	157 ± 8	54 ± 1	nd
ΨMG14	118 ± 11	25 ± 2	nd
ΨMG15	112 ± 7	17 ± 0	nd
pHa-MDR-IRES-αGal(2)			
ΨMG21	335 ± 46	127 ± 8	202
ΨMG22	251 ± 43	85 ± 11	67
ΨMG23	157 ± 11	76 ± 3	nd
ΨMG24	181 ± 5	71 ± 3	56
ΨMG25	150 ± 10	70 ± 3	nd
ΨMG26	108 ± 3	40 ± 3	nd
ΨMG27	115 ± 14	26 ± 1	nd

[a]Standard deviations are shown.
[b]Mean channel number in FACS fluorogram.
[c]nd, Not determined.

TABLE 4. α-GAL A ACTIVITY, VINCRISTINE SENSITIVITY, AND P-GLYCOPROTEIN EXPRESSION OF NIH-3T3 POPULATIONS TRANSDUCED WITH THE *MDR*1 VECTORS

	α-Gal A activity (% of control)[a]	*IC_{50} to vincristine (ng/ml)*[a]	*P-glycoprotein expression*[b]
NIH-3T3	100 ± 4	3.8 ± 0.2	2
pHaMDR	96 ± 3	78 ± 9	53
pHa-MCS-IRES-MDR	96 ± 7	61 ± 3	19
pHa-αGal-IRES-MDR	1,034 ± 167	31 ± 6	9
pHa-MDR-IRES-αGal(1)	469 ± 24	78 ± 4	71
pHa-MDR-IRES-αGal(2)	488 ± 58	76 ± 7	69

[a]Standard deviations are shown.
[b]Mean channel number in FACS fluorogram.

Coexpression of P-glycoprotein and α-Gal A after retrovirus transduction

Next we determined the activity of α-Gal A in the mixed populations of NIH-3T3 cells transduced with the *MDR*1 retrovirus. The α-Gal A activity, the IC_{50} for vincristine, and the mean channel of the fluorescence-activated cell sorting (FACS) fluorogram for each population are shown in Table 4. Again, the pHa-αGal-IRES-MDR transductants and pHa-MDR-IRES-αGal transductants showed high α-Gal A activity and P-glycoprotein expression when compared to the parental NIH-3T3 cells. In this experiment, apparently greater increases in α-Gal A activity were observed than in the results of Table 3. This is mostly because of the difference in the endogenous enzyme activities. The endogenous α-Gal A activities (pmol/min · mg protein) in Ψ-cre and NIH-3T3 cells were 490 and 150, respectively.

We then isolated NIH-3T3 clones transduced with the *MDR*1 retrovirus and measured the α-Gal A activity and P-glycoprotein expression. As shown in Table 5, all four clones (NGM1–NGM4) transduced with pHa-αGal-IRES-MDR showed 15- to 20-fold higher α-Gal A activity than the parental NIH-3T3 cells. These transductants also showed six- to nine-fold higher resistance to vincristine than the parental cells. The expression of human P-glycoprotein in the transductants was confirmed by FACS analysis. This result clearly demonstrates that the transferred retrovirus results in coexpression of two genes in the transduced cells. All four clones (NMG11, NMG12, NMG21, and NMG22) transduced with pHa-MDR-IRES-αGal showed higher α-Gal A activity than the parental NIH-3T3 cells. Again, the pHa-MDR-IRES-αGal transductants showed higher levels of drug resistance (13- to 19-fold) and lower levels of α-Gal A activity (4- to 8-fold) than the pHa-αGal-IRES-MDR transductants.

TABLE 5. α-GAL ACTIVITY, SENSITIVITY TO VINCRISTINE, AND P-GLYCOPROTEIN EXPRESSION OF NIH-3T3 CLONES TRANSDUCED WITH THE VECTORS

	α-Gal A activity (% of control)[a]	*IC_{50} to vincristine (ng/ml)*[a]	*P-glycoprotein expression*[b]
NIH-3T3	100 ± 4	3.6 ± 0.32	2
pHaMDR			
NM1	93 ± 7	75 ± 4	63
NM2	94 ± 1	71 ± 3	66
pHa-MCS-IRES-MDR			
NIM1	103 ± 4	53 ± 8	26
NIM2	96 ± 3	55 ± 0	22
pHa-αGal-IRES-MDR			
NGM1	1,966 ± 135	35 ± 2	19
NGM2	1,820 ± 236	31 ± 1	16
NGM3	1,535 ± 62	28 ± 0	12
NGM4	1,471 ± 90	25 ± 3	14
pHa-MDR-IRES-αGal(1)			
NMG11	767 ± 40	75 ± 3	60
NMG12	412 ± 29	60 ± 1	25
pHa-MDR-IRES-αGal(2)			
NMG21	609 ± 49	70 ± 3	55
NMG22	368 ± 21	53 ± 2	30

[a]Standard deviations are shown.
[b]Mean channel number in FACS fluorogram.

Western blot analysis

To determine whether the *MDR*1 cDNA and α-Gal A cDNA were efficiently translated from the bicistronic mRNAs to yield detectable amounts of protein, we performed Western blot analysis on cell extracts from vincristine-resistant NIH-3T3 clones transduced with the vectors. The expression of P-glycoprotein was higher in all the transfectants than in the parental NIH-3T3 cells (Fig. 3A). The size of P-glycoprotein expressed under control of the IRES (clone NIM1, NGM1, and NGM2) was the same as that expressed in pHaMDR-transduced cells (clone NM1). A clone (NM1) transduced with pHaMDR and three clones (NMG11, NMG21, and NMG22) transduced with pHa-MDR-IRES-αGal showed high expression of P-glycoprotein. Two clones, NGM1 and NGM2, transduced with pHa-αGal-IRES-MDR expressed only small amounts of P-glycoprotein. In contrast, high-level expression of the human 52-kD α-Gal A was detected in two pHa-αGal-IRES-MDR-transduced clones, NGM1 and NGM2 (Fig. 3B). Three pHa-MDR-IRES-αGal-transduced clones also expressed higher amounts of α-Gal A than the parental NIH-3T3 cells or control *MDR*1-transduced cells (Fig. 3B).

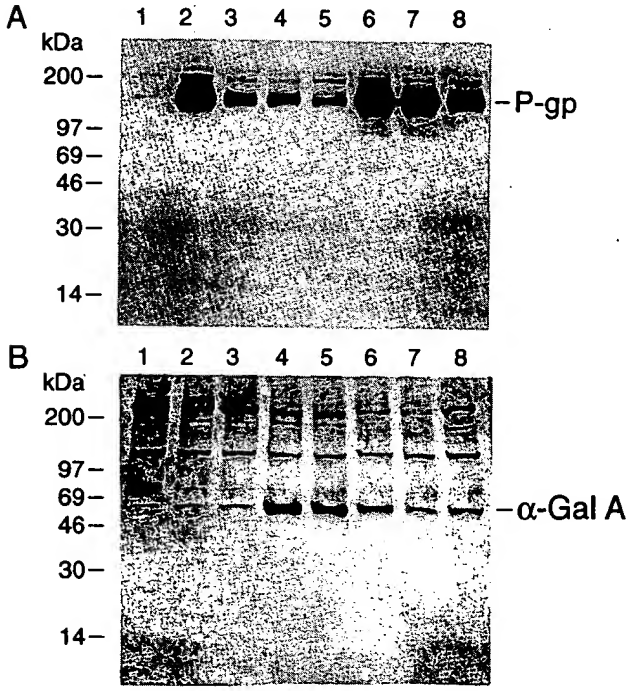

FIG. 3. Western blot analyses of P-glycoprotein and α-Gal A in NIH-3T3 clones transduced with pHaMDR, pHa-MCS-IRES-MDR, pHa-αGal-IRES-MDR, and pHa-MDR-IRES-αGal. Cell extracts (40 μg per lane) prepared as described in Materials and Methods were fractionated by 4–20% SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting. A. Detection of P-glycoprotein using C219 monoclonal antibody and peroxidase-conjugated goat anti-mouse IgG. B. Detection of α-Gal A using rabbit anti-human α-Gal A antibody and peroxidase-conjugated goat anti-rabbit IgG. The peroxidase on the blots was visualized using the ECL chemiluminiscence detection kit. Lane 1, Untransduced NIH-3T3 control; lane 2, pHaMDR-transduced clone NM1; lane 3, pHa-MCS-IRES-MDR-transduced clone NIM1; lanes 4 and 5, pHa-αGal-IRES-MDR-transduced clones NGM1 and NGM2, respectively; lane 6, pHa-MDR-IRES-αGal(1)-transduced clone NMG11; lanes 7 and 8, pHa-MDR-IRES-αGal(2)-transduced clones NMG21 and NMG22, respectively. P-gp, P-glycoprotein; αGal A; α-Gal A. The sizes of molecular weight markers (kilodaltons) are indicated.

DISCUSSION

In this study, we developed bicistronic retroviral vectors for the coexpression of the human *MDR*1 gene and a second nonselectable gene (α-Gal A). We have recently created a similar bicistronic retroviral vector in a somewhat less flexible system for coexpression of the upstream *MDR*1 and the downstream glucocerebrosidase and demonstrated the colchicine-selected coexpression of human glucocerebrosidase in NIH-3T3 cells using DNA transfection (Aran *et al.*, 1994). In the current study, we examined the effectiveness of two types of bicistronic constructs using both DNA transfection and retrovirus-mediated gene transfer. Higher activity of α-Gal A was consistently seen in pHa-αGal-IRES-MDR-transfected cells than in pHa-MDR-IRES-αGal-transfected cells, but higher expression of the *MDR*1 gene was observed in pHa-MDR-IRES-αGal-transfected cells than in pHa-αGal-IRES-MDR-transfected cells. An amphotropic retrovirus titer of 3×10^4 was obtained with pHa-αGal-IRES-MDR, and a titer of 1.2×10^5/ml was obtained with pHa-MDR-IRES-αGal in the PA317 packaging cell line.

The pHa-αGal-IRES-MDR and pHa-MDR-IRES-αGal constructs were able to confer multidrug resistance when introduced into drug-sensitive cells and to be packaged as retroviruses. In both transfection and transduction experiments, numbers of drug-resistant colonies were pHaMDR > pHa-MDR-IRES-αGal > pHa-MCS-IRES-MDR > pHa-αGal-IRES-MDR. One reason for the slightly lower activity of pHa-MDR-IRES-αGal than that of pHaMDR may be that pHa-MDR-IRES-αGal has an insert 1.8-kb longer than that of pHaMDR. Since pHa-αGal-IRES-MDR has the same components in its insert (α-Gal A, IRES, and *MDR*1) as pHa-MDR-IRES-αGal, the decreased ability of pHa-αGal-IRES-MDR to confer multidrug resistance is due to the difference in the sites where the *MDR*1 cDNAs are inserted. Therefore, it seems reasonable to conclude from our results that translation of the *MDR*1 cDNA under the control of the IRES is less efficient than cap-dependent translation.

When we examined the drug resistance and P-glycoprotein expression of the transfectant or transductant clones, the diversity among the clones was less in cells carrying pHa-αGal-IRES-MDR than in cells with pHa-MDR-IRES-αGal. The pHa-αGal-IRES-MDR transfectants showed IC_{50} values to vincristine ranging from 14 to 29 ng/ml (Table 4). It should be noted that these clones were selected with 25 ng/ml of vincristine. Transfectant clones which show IC_{50} values for vincristine less than 14 ng/ml would probably be killed under this selection condition. Because fewer drug-resistant colonies were obtained from the pHa-αGal-IRES-MDR-transfected cells than from the pHa-MDR-IRES-αGal-transfected cells, the vincristine selection was probably more stringent for the pHa-αGal-IRES-MDR-transfected cells, and allowed for the survival only of the clones expressing relatively large amounts of bi-

cistronic mRNA. This would account for why all the clones carrying pHa-αGal-IRES-MDR showed a substantial increase in α-galactosidase A activity.

In contrast, we observed large differences in drug resistance and α-Gal A activity among the clones carrying pHa-MDR-IRES-αGal. The pHa-MDR-IRES-αGal transfectants or transductants with high levels of drug resistance also showed larger amounts of α-Gal A activity. In the results presented in Table 3, only 8/12 transfectant clones showed substantial increases in α-Gal A activity compared to the parental Ψ-cre cells. When the α-Gal A activity in each transfectant clone is compared to the IC_{50} values for vincristine of the clone, pHa-MDR-IRES-αGal-transfected Ψ-cre cells show significantly higher α-Gal A activity only when the cells show high levels of drug resistance (the IC_{50} values are more than 50 ng/ml). Every one of the four transfectant clones with IC_{50} values for vincristine of less than 40 ng/ml did not show substantial increases in α-Gal A activity. As discussed above, the pHa-MDR-IRES-αGal transfectants could probably form drug-resistant colonies even though mRNA expression was low, because these cells can translate the *MDR*1 cDNA more efficiently than the pHa-αGal-IRES-MDR transfectants. Another likely reason for the low activity of α-Gal A in pHa-MDR-IRES-αGal transfectants is that the α-Gal A gene in the construct is less effectively translated than in pHa-αGal-IRES-MDR because it is translated under the control of the IRES. Since the pHa-MDR-IRES-αGal constructs contain minor changes in their signal sequences (see Table 1), the reduction in α-Gal A activity might be related to these changes, but it is unlikely because α-Gal A activities seem to parallel amounts of α-Gal A protein detected on Western blots (Fig. 3). It should be noted that DNA rearrangements sometimes occur in retrovirus-mediated gene transfer (Olsen *et al.*, 1993). Therefore, it is possible that the lack of α-Gal overexpression in some of pHa-MDR-IRES-αGal transfects may be due to DNA arrangements.

In separate work, we examined the probability of the coexpression of two genes using a similar retroviral construct, pHa-MDR-IRES-TK (Sugimoto *et al.*, 1995). In this construct, both the *MDR*1 gene and the herpes simplex virus thymidine kinase (HSV-TK) gene can be used as drug-selectable markers when a thymidine-kinase-deficient cell line Ltk^- is used as the recipient. In that study, when the transfected cells were selected in HAT medium based on expression of the HSV-TK gene (downstream from the IRES), each of the 20 clones examined expressed both genes. When the cells were selected by expression of the *MDR*1 gene (upstream from the IRES), 19 clones out of 20 expressed both genes (Sugimoto *et al.*, submitted for publication). In the current study, we could use only the *MDR*1 gene as a drug-selectable marker, but as seen in Tables 3 and 5, the same pattern holds in the pHa-MDR-IRES-αGal vectors. Based on the αGal-MDR and the MDR-TK vectors, it appears that up to 95% of the drug-resistant cells express significant amounts of the upstream nonselected cDNAs when the drug-selectable markers are inserted downstream from the IRES.

Because we have observed higher levels of expression of the genes inserted upstream, rather than downstream, from the IRES, this vector system should make it possible to express different ratios of drug resistance to nonselectable gene expression, depending on which gene is upstream from the IRES and which is downstream. Further flexibility might be achieved by exploiting the polarity in expression afforded by the IRES, by addition of additional spacer sequences and IRES sequences upstream from the second gene. For the purpose of gene therapy of Fabry disease, we will need to determine whether the ability of pHa-αGal-IRES-MDR to express *MDR*1 is enough to confer drug resistance *in vivo* or not.

The differences in α-Gal A activities among the clones of the vincristine-selected pHa-α-Gal-IRES-MDR transfectants or transductants were less than two-fold. Therefore, by using similar polycistronic constructs, it should be possible to control the expression level of the foreign gene in the transduced cells so that all the cells express an appropriate amount of the gene. This is a desirable feature for a vector system if the expression of the transduced gene at an undesired level is toxic to cells. The parallel expression of the gene of interest with cell-surface P-glycoprotein enables the removal of cells expressing undesirable amounts of the gene of interest using cell sorting or panning methods *ex vivo* with monoclonal antibodies to P-glycoprotein. Protocols are already established to isolate *MDR*1-expressing cells from a heterogeneous cell population by FACS (Choi *et al.*, 1991) or by magnetic-affinity cell sorting (Padmanabham *et al.*, 1991). This approach could also be used to select *ex vivo* for cells expressing high levels of P-glycoprotein and a second gene of interest, possibly obviating or eliminating the need for selection with toxic drugs *in vivo*. Polycistronic vector systems that employ *MDR*1 as a selectable marker appear to be flexible and efficient vehicles for introduction of nonselectable genes into cells.

ACKNOWLEDGMENTS

We would like to thank Drs. Takashi Tsuruo and Kenichi Ono for generously providing the MRK16 antibody, Dr. Richard C. Mulligan for Ψ-cre and Ψ-crip cells, and Drs. Alfred C. Johnson and Christine Hrycyna for helpful discussions.

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Received for publication August 12, 1994; accepted after revision March 16, 1995.

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CODEN: HGTHE3; ISSN: 1043-0342

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Biochemical Analysis of the 3460, 11778, and 14484 Leber's Hereditary Optic Neuropathy (LHON) mtDNA Mutations in Lymphoblasts and Transmitochondrial Cybrids. M.D.Brown, I.Trounce, B.Jun, A.Panov, and D.C. Wallace. Department of Genetics and Molecular Medicine, Emory University School of Medicine, Atlanta, GA.

To investigate the pathophysiological mechanism of LHON, we studied respiration and mitochondrial enzyme activities in lymphoblasts and lymphoblast-derived transmitochondrial cybrids for the 3 "primary" LHON mtDNA mutations at nucleotide positions 3460, 11778, and 14484. Each of these mutations alters a polypeptide constituent of Complex I (NADH:ubiquinone oxidoreductase).

For respiration studies in lymphoblasts, state III rates using NADH-linked substrates were reduced for all three mutations, although most markedly in 11778 mitochondria, in which the state III rate was 30% lower than controls. Uncoupled rates were generally reduced for all three LHON genotypes, but ADP/O ratios did not differ significantly from control ratios. Similar results were observed in cybrid cell lines, but with less marked differences between patients and controls. For enzymological analysis in lymphoblasts, Complex I activity was reduced for all mutations (3460 = 70% reduction relative to cybrid controls, 11778 = 30% reduction, 14484 = 20% reduction). Citrate synthase (CS) normalized Complex I activities exhibited the same degree of dysfunction. There were essentially no differences between patients and controls for Complex II+III, III, or CS assays, although Complex IV activity was elevated in 3460 and 11778 mitochondria. In cybrids, an enzymatic defect was only seen for the 3460 mutation, which exhibited a 73% reduction in absolute and normalized Complex I activities.

This study represents the first time that a direct comparison of the biochemical phenotypes of the important LHON mutations can be made. Using a single tissue and with uniform assays, we have shown that: (1) Complex I defects were found for all three mutations, with the most marked respiration defect found in 11778 mitochondria and the most marked enzymological defect found in 3460 mitochondria, (2) the 14484 mutation has the most benign biochemical phenotype, and (3) cybrid transfer tended to reduce differences between patient and controls values relative to the lymphoblast data, thus demonstrating the influence of the nuclear genetic background. We are currently investigating the kinetic parameters of mutant Complex I in an attempt to identify a common pathophysiological mechanism for LHON.

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American J. of Human Genetics, 1996, Vol. 59 No. 4

L - [1 ^{13}C]-phenylalanine oxidation in hyperphenylalaninemia (HPA): analysis of genotype/phenotype correlations. C.R. Scriver[1], J.J. Delvin[2], G. Eisa[1], K. Cotter[1], M. Lambert[2], P. Waters[1], E.P. Treacy[2]. [1]Dept. of Human Genetics, McGill Univ.-Montreal Children's Hospital, Canada, [2]Martek Biosciences Corp., Columbia, MD and [3]Dept. of Genetics, Hôp. Ste-Justine, Montreal, Canada.

Hyperphenylalaninemia (HPA) results from deficiency of the enzyme phenylalanine hydroxylase, with 302 known mutations at the *PAH* locus (McKusick 261600; http://www.mcgill.ca/pahdb), including 33 that have been expressed *in vitro*. Hypothesis: The *in vivo* metabolic counterpart (HPA) is an emergent property and may not correlate consistently with the *in vitro* unit-protein (enzymic) phenotype. Methods: i) We developed a novel, rapid (60 min), non-invasive breath test to measure whole-body phenylalanine oxidation from cumulative $^{13}CO_2$ recovered at 60 min, (isotopic plateau) in: 7 classic PKU (phe tolerance < 500 mg); 6 variant PKU (phe tolerance > 500 mg/day); 4 non-PKU HPA subjects; 17 obligate heterozygotes; and 8 controls. We correlated *in vivo* data with genotype (*PAH* mutations analyzed by DGGE and DNA sequencing) and *in vitro* expression data (where known). We also compared the density function for noon-time plasma phenylalanine level and the phenylalanine/tyrosine ratio with $^{13}CO_2$ data in obligate heterozygotes. Results: (i) Oxidation data (% ^{13}C oxidized), differed between groups (mean ± SD): all HPA cases, 0.29±0.58; heterozygotes 4.77±1.88; controls, 7.95±1.43; p <0.001; ii) differences existed between the subclasses of HPA; iii) heterozygotes harbouring 'severe' and 'mild' *PAH* mutations could be differentiated; iv) discrepancies were noted between observed (*in vivo*) oxidation rates and predicted effect (*in vitro* expression) of alleles. Conclusion: The ^{13}C phenylalanine breath test is a reproducible non-invasive rapid measure of *in vivo* phenylalanine oxidation. It can discriminate between mutant, homo- and heteroallelic phenotypes of differing severity; and between the heterozygous and wild type. HPA has both Mendelian (major locus) and emergent (complex trait) properties.

69

Possibility of gene therapy for globoid cell leukodystrophy (GLD, Krabbe disease) is indicated by successful transfer of GALC activity from overexpressing cells to deficient cells. M.A. Rafi[1], J. Fugaro[1], T. Victoria[1], S. Amini[1], P. Luzi[1], A. Beggs[2], H.Q. Tong[2] and D.A. Wenger[1]. [1]Jefferson Medical College, Philadelphia, PA and [2]Children's Hospital, Boston, MA.

Krabbe disease or GLD is an autosomal recessive disorder caused by the deficiency of galactocerebrosidase (GALC) activity. This enzyme catalyzes the lysosomal hydrolysis of galactosylceramide, an important component of CNS and PNS myelin. Two animal models, twitcher mice and terrier dogs, are well characterized and available for gene therapy trials. GALC is extremely hydrophobic, existing in very low endogenous levels as a high molecular weight complex (>700 kD) composed of its 30 and 50 kD subunits. Human GALC cDNA with a modified start site was placed in the MFG retroviral vector, and used to transfect ψ-crip cells. The MFG-GALC from the packaging cell line successfully transduced many cell types including fibroblasts from patients and dogs with GLD, human CD34+ hematopoietic cells, rat brain astrocytes, mouse Schwann cells, and myoblasts from twitcher mice. Deficient cells reached GALC levels 20,000 times pre- infection levels (about 100 times normal) with no ill effects. These cells secreted GALC into the media that was efficiently taken up by other deficient cells. Normal values were obtained in recepient cells in 2-3 days. This compares to the very low transfer of GALC activity by untransduced normal cells. The high uptake of GALC from media was inhibited 50% by mannose-6-P indicating that significant uptake was via a non-receptor-mediated mechanism, probably pinocytosis. Astrocytes and Schwann cells were also successfully transduced to levels 10-20 times baseline by this vector, and these cells were able to donate GALC to deficient fibroblasts with high efficiency. As bone marrow transplantation of affected patients using a normal donor has been demonstrated to have a positive, but not curative, effect on the clinical course, it is postulated that transplantation of cells overexpressing GALC would provide significantly more enzyme for delivery to neighboring cells.

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CORRECTION OF ORNITHINE ACCUMULATION PREVENTS RETINAL DEGENERATION IN A MOUSE MODEL OF GYRATE ATROPHY OF THE CHOROID AND RETINA Tao Wang, Ann H. Milam, and David Valle. Howard Hughes Medical Institute and the Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore; Department of Ophthalmology, University of Washington School of Medicine, Seattle.

Deficiency of ornithine-δ-aminotransferase (OAT) in humans results in gyrate atrophy (GA), an autosomal recessive disorder characterized by hyperornithinemia and a slowly progressive chorioretinal degeneration. To develop a mouse model for study of the pathogenesis and treatment of GA, we produced an OAT-deficient mouse by gene targeting. Post-weaning, $OAT^{-/-}$ mice develop hyperornithinemia similar to human GA patients (plasma ornithine mean ± SD: $OAT^{-/-}$ mice, 1172 ± 251; normal mice, 95 ± 21) and a slowly progressive retinal degeneration. Mean electroretinogram (ERG) amplitude is normal at 2 months but reduced to 40% by 12 months. At 2 months the retinas of $OAT^{-/-}$ mice are normal histologically but by 7 [illegible]ths the RPE cells are swollen with loss of basal infoldings and apical microvilli and the photoreceptor outer segments are highly disorganized and reduced in length. Retinal amino acids show ornithine accumulation 15 x control. To determine the effect of chronic reduction of ornithine on the retinal phenotype, we placed $OAT^{-/-}$ mice on an arginine-restricted diet at 2 months of age. Plasma ornithine was reduced to near control levels (mean 167, range 74-249) and normal growth was maintained.. At 12 months, ERG amplitudes and retinal histology of the treated $OAT^{-/-}$ mice were normal. We conclude that the $OAT^{-/-}$ mice are an excellent biochemical and retinal model of GA; that ornithine accumulation is central to the pathophysiology; and, that chronic, systemic correction of ornithine accumulation prevents the retinal degeneration.

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Fabry disease: enzyme replacement therapy in α-galactosidase A deficient mice. Y.A. Ioannou[1], K.M. Zeidner[1], B. Friedman[2] and R.J. Desnick[1]. 1 Department of Human Genetics, Mount Sinai School of Medicine, NY 2 Genzyme Corporation. Framingham, MA

Fabry disease is an X-linked lysosomal storage disorder that results from the deficient activity of the lysosomal hydrolase α-galactosidase A (α-Gal A). The progressive accumulation of its major glycolipid substrate, globotriaosylceramide (GL-3), particularly in vascular lysosomes, results in premature death due to vascular disease of the kidney, heart or brain. The recent availability of mice with α-Gal A deficiency, generated by gene targeting, permits the evaluation of the potential therapeutic effectiveness of α-Gal A replacement. Studies were conducted in the enzyme deficient mice to determine the plasma clearance and biodistribution of four different α-Gal A glycoforms which varied in their levels of sialylation. The plasma clearance half-lives for all four recombinant glycoforms were about 3 to 5 min, with the most highly sialylated glycoform having the longest half-life. All four glycoforms were primarily taken up by the liver, with a small amount present in kidney and spleen 1 hr after intravenous administration. The *in vivo* stability was evaluated for two of the α-Gal A glycoforms; one that was sialylated and one that was non-sialylated. Ninety-six hours after administration, the amount of α-Gal A remaining in the liver of each of these two glycoforms was 10% and 5% respectively. This relatively long stability is consistent with the enzyme being localized in lysosomes. To assess the effect of enzyme replacement on the accumulated substrate, an initial series of four intravenous injections of $5X10^5$ units (nmol/hr) of the sialylated glycoform of α-Gal A was administered at 48 hr intervals into each of four mice. Compared to untreated littermates, the GL-3 levels in plasma, liver, and heart were markedly decreased, while only a slight decrease was observed in the kidney. However, a series of eight injections of the same dose at 48 hr intervals decreased the levels of renal GL-3 by 30-50%. Ultrastructural examination of liver revealed empty vacuoles indicating that the administered enzyme gained access to and hydrolyzed the accumulated substrate. These preclinical results provide the rationale for enzyme replacement in patients with Fabry disease.

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Amelioration of the skeletal disease in hypophosphatasia by bone marrow transplantation using the alkaline phosphatase-knockout mouse model. K.N. Fedde[1], L. Blair[1], F. Terzic[1], H.C. Anderson[2], S. Narisawa[3], J.L. Millan[3], M.P. Whyte[1,4]. Washington Univer Schl of Med, St. Louis, MO[1]; Univer of Kansas Med Center[2], The Burnham Institute, La Jolla, CA[3]; and Shriners Hospital for Crippled Children, St. Louis, MO[4].

Hypophosphatasia (HPP) is an untreatable autosomal recessive skeletal disease (rickets/osteomalacia) resulting from mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene and deficient TNSALP activity. *Infantile* HPP typically manifests with progressive skeletal disease developing during the first 6 months-of-life with lethality reported in 50% of patients. Therapeutic trials by intravenous infusion of ALP appear to be unsuccessful. Accordingly, we investigated a TNSALP gene-knockout (null allele) mouse as a model for HPP and tested the therapeutic efficacy of bone marrow transplantation.

The HPP mouse (-/-) has absent TNSALP mRNA and TNSALP activity (all isoforms; bone, liver, kidney). Radiographs of lower limbs show skeletal disease in -/- mice by ~10 days-of-age with rachitic changes and failure of appearance of 2° ossification centers. Noted absence of peritrabecular fibrosis, osteoclastosis, or excessive plump osteoblasts is evidence against underlying 2° hyperparathyroidism. Plasma and urine calcium levels are normal in -/- and +/- mice, also arguing against secondary causes for the rachitic disease.

Bone marrow cells (from +/+ ♀'s) were injected ($1.5x10^6$ cells) intravenously into newborn HPP mice after irradiation. Transplanted HPP mice were compared to PBS-injected, irradiated control and HPP mice. Semi-quantitative ALP histochemistry of blood smears from transplanted HPP mice indicated the presence of donor leukocytes; we estimated ~35% engraftment. From 11 to 44 day-of-age, radiographic studies showed amelioration of the skeletal disease in all 13 transplanted HPP mice.

We find that the TNSALP gene-knockout mouse appears to be a good model for infantile HPP. Using the HPP mouse model, these preliminary observations provide evidence for the efficacy of bone marrow transplantation for inborn errors of osteoblast function.

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Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7917–7922, July 1996
Medical Sciences

Correction in trans for Fabry disease: Expression, secretion, and uptake of α-galactosidase A in patient-derived cells driven by a high-titer recombinant retroviral vector

JEFFREY A. MEDIN*†, MATTHEW TUDOR*, RYAN SIMOVITCH*, JANE M. QUIRK*, STEVEN JACOBSON‡, GARY J. MURRAY*, AND ROSCOE O. BRADY*

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Contributed by Roscoe O. Brady, April 23, 1996

ABSTRACT **Fabry disease is an X-linked metabolic disorder due to a deficiency of α-galactosidase A (α-gal A; EC 3.2.1.22). Patients accumulate glycosphingolipids with terminal α-galactosyl residues that come from intracellular synthesis, circulating metabolites, or from the biodegradation of senescent cells. Patients eventually succumb to renal, cardio-, or cerebrovascular disease. No specific therapy exists. One possible approach to ameliorating this disorder is to target corrective gene transfer therapy to circulating hematopoietic cells. Toward this end, an amphotropic virus-producer cell line has been developed that produces a high titer (>10^6 i.p. per ml) recombinant retrovirus constructed to transduce and correct target cells. Virus-producer cells also demonstrate expression of large amounts of both intracellular and secreted α-gal A. To examine the utility of this therapeutic vector, skin fibroblasts from Fabry patients were corrected for the metabolic defect by infection with this recombinant virus and secreted enzyme was observed. Furthermore, the secreted enzyme was found to be taken up by uncorrected cells in a mannose-6-phosphate receptor-dependent manner. In related experiments, immortalized B cell lines from Fabry patients, created as a hematologic delivery test system, were transduced. As with the fibroblasts, transduced patient B cell lines demonstrated both endogenous enzyme correction and a small amount of secretion together with uptake by uncorrected cells. These studies demonstrate that endogenous metabolic correction in transduced cells, combined with secretion, may provide a continuous source of corrective material in trans to unmodified patient bystander cells (metabolic cooperativity).**

Corrective gene transfer has been suggested as curative therapy for a number of human disorders that result from single enzyme defects. This strategy is especially attractive in lysosomal storage disorders that involve elements from the hematopoietic system (1–4). Indeed, Fabry disease is a compelling model to examine approaches to deliver corrective factors to compromised cells and tissues because no specific therapy currently exists despite the identification of the enzymatic defect in Fabry disease in 1967 (5).

Fabry disease is an X-linked recessive panethnic disorder caused by a deficiency of the enzyme α-galactosidase A (α-gal A). Patients with diminished enzyme activity accumulate high levels of incompletely metabolized glycosphingolipids that have terminal α-galactosyl residues (for review, see ref. 6). These lipids are deposited in many systemic organs as well as blood vessels and neurons (7). Patients have characteristic angiokeratoma, hypohidrosis, and episodic pain crises in the extremities, and they eventually succumb to renal disease, myocardial infarction, or stroke.

This study describes the construction and application of a high-titer recombinant retroviral vector to correct the enzymatic defect in hematopoietic and other cells derived from patients with Fabry disease. Moreover, cells engineered to overexpress α-gal A by retroviral transduction were found also to secrete the specific enzyme activity. Secreted α-gal A activity was found to be able to be taken up by uncorrected patient's cells in a mannose-6-phosphate (Man-6-P) receptor-dependent manner. These findings enhance prospects of therapy for Fabry disease by gene correction in concert with enzymatic correction of unmodified bystander cells.

MATERIALS AND METHODS

Vector Construction. The full-length α-galactosidase A cDNA was amplified by PCR from plasmid pGB78A using DNA oligonucleotide primers that added unique *Not*I and *Xho*I (New England Biolabs) restriction enzyme sites at the 5′ and 3′ noncoding ends of the sequence. The sequences of the primers GalA1 and GalA2 were 5′-GATCCTTTGCGGCCGC-3′ and 5′-CCTGGGCTCGAGTTAAAGTAAGTCTTTTAATG-3′, respectively. The resulting PCR product was digested and subcloned into retroviral vector PG1 (Genetic Therapy, Gaithersburg, MD) at the *Not*I and *Xho*I sites and sequenced to insure fidelity. Initial sequence results demonstrated no nucleotide alterations in the PCR product from the wild-type α-gal A cDNA sequence. However, repeated intensive sequencing showed that a single G nucleotide at position 531 was changed to an A. This alters the amino acid ^{178}Glu to a lysine residue. Contextual analyses predicted that this alteration would have little effect on the predicted hydrophilicity, surface probability, chain flexibility, helical structure, and antigenicity index for that region.

The PG1α-gal A DNA plasmid construct was then transfected into BOSC23 cells according to Pear *et al.* (8) and ecotropic retroviral supernatant was collected from multiple plates at 24 and 48 hr after transfection. The supernatant solution was used to infect amphotropic virus-producing GP + AM12 packaging cells (9) multiple times in the presence of polybrene (Sigma). The pool of infected AM12 cells was used as a producer virus source in some initial studies. Amphotropic virus-producing cell clones were also expanded from isolated single cells and assayed for α-gal A enzyme activity (see below). A single clone, AM12PG1α-gal A#8, was selected for subsequent studies.

Cell Culture, Virus Production, and Infection. GP + AM12, NIH 3T3 (American Type Culture Collection), and BOSC23 cells, were maintained under standard culture conditions. Cultured skin fibroblasts and leukocytes from peripheral blood from patients with Fabry disease were collected under approved National Institutes of Health protocols. Immortalized B cell lines were generated and maintained after Epstein–Barr virus (EBV) transformation of leukocytes derived from peripheral blood as described (10). The AM12PG1α-gal A#8

Abbreviations: α-gal, α-galactosidase; Man-6-P, mannose-6-phosphate.

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packaging cell construct was tested by sensitive marker rescue assays for the presence of helper virus (10). No helper virus was found at any dilution (data not shown). Viral infections of all target cells with viral supernatants were performed essentially as described (10). Briefly, cells as monolayers on plates or as suspension cultures, were infected with filtered supernatant from the AM12PG1α-gal A#8 producer line which had been incubated 16 hr previously with the media specific to the target cells of interest. Filter-sterilized protamine sulfate (Elkins–Sinn, Cherry Hill, NJ) at 4 μg/ml was added to each incubation. Virus (as supernatant) was added to target cells for four consecutive overnight applications.

Southern Blot Analysis. Genomic DNA, prepared from NIH 3T3 cells infected with supernatant from the AM12PG1α-gal A#8 producer line using an isolation kit (Qiagen, Chatsworth, CA) and the manufacturer's protocols, was digested with restriction enzyme *Nhe*I (New England Biolabs) and separated by electrophoresis on agarose gels. A single provirus band was detected after transfer to membranes and hybridization to a nonradioactive reporter system (Tropix, Bedford, MA). An effective viral titer of $>5 \times 10^6$ infectious units/ml was observed from two separate assays (data not shown).

Enzyme Assays. The fluorimetric assay of α-gal A activity (11) was modified as follows. Retroviral producer cells or confluent fibroblasts, harvested by cell scraping, or leukocytes and immortalized B cells, were pelleted by low-speed centrifugation. The cell pellets were washed 3 times with PBS, sonicated 2 × 5 sec on ice in homogenization buffer (28 mM citric acid/44 mM disodium phosphate/3 mg/ml sodium taurocholate, pH 4.4) and then centrifuged at 20,000 × *g* for 30 min. Total α-gal activity was determined by incubating aliquots of the supernatant solutions for various times with 10 mM 4-methylumbelliferyl-α-D-galactopyranoside (Research Products International) in the homogenization buffer at 37°C without taurocholate but with bovine serum albumin (5 mg/ml). Media was collected, filtered, and assayed directly. Isoenzyme-specific α-gal A activity was determined by comparison of total α-gal activity with that observed in the presence of 0.1 M *N*-acetylgalactosamine, an inhibitor of α-gal B (12). As an additional control for specificity, β-hexosaminidase activity was measured in cell extracts and media (13). One unit of α-gal A activity is equivalent to the hydrolysis of 1 nmol of substrate in 1 hr at 37°C.

Uptake and Secretion Studies. Fresh media, appropriate to the type of recipient cell being examined, were added to source cells 12–16 hr before assay. This media was then collected and filtered through a 0.45 μm filter. The media was then assayed as above or added to recipient cells, which were then incubated for 3 hr at 37°C or at 4°C in the presence or absence of 1 mM Man-6-P. The recipient cells were then collected and assayed as above.

RESULTS

The Retroviral PG1α-Gal A Construct. A recombinant vector was designed for eventual therapy trials in patients with Fabry disease. Recombinant virus produced from the PG1 backbone, used in the present study, has been shown to have high titer (14) and lead to stable production of a corrective factor over time following provirus integration (15).

Intracellular α-gal A activity was measured in the total cell pool and in numerous AM12PG1α-gal A cell clones isolated after multiple infections with the transfected BOSC23 ecotropic viral supernatant. Specific activities for various clones were increased from 10- to 50-fold over background AM12 levels. The highest level of enzyme activity approximated 10^5 units/mg cell protein (data not shown). Based on this information, a single clone was selected for subsequent study. The effective retroviral titer of this clone was estimated by supernatant infection of NIH 3T3 cells followed by semiquantitative Southern blot analyses. In two independent assays, a titer of $>5 \times 10^6$ infectious particles per ml was observed (data not shown).

AM12PG1α-gal A#8 producer cells were also examined for the secretion of α-gal A activity. No secretion of α-gal A activity was observed from uninfected AM12 cells. In contrast, $\approx 2 \times 10^6$ AM12PG1α-gal A#8 cells secreted ≈100 nmol/hr/ml of α-gal A activity into the media (data not shown). This level represents ≈0.05 mg of α-gal A enzyme per liter of supernatant, based on a minimum specific activity of 2×10^6 units/mg for the purified enzyme (G.J.M., unpublished observations). No secreted β-hexosaminidase activity was measurable in the media of either parent AM12 cells or the producer cell clone (data not shown) indicating that this was not a broad lysosomal enzyme effect.

Enzymatic Correction of Cells Obtained from Patients with Fabry Disease. Cultured skin fibroblasts ($\approx 2 \times 10^6$ cells), derived from a moderately affected patient with Fabry disease, were infected four times with supernatant from the AM12PG1α-gal A cell pool. α-Gal A specific enzyme activity was then measured in the collected cells. Uncorrected cells exhibited an α-gal A enzyme specific activity of 210 units/mg cell protein (≈29% of normal; Fig. 1). After transduction with supernatant from the uncloned AM12PG1α-gal A producer cell pool, α-gal A activity increased to more than 10-fold above the normal level (Fig. 1). No increase in α-gal A activity was observed in the mock-infected cells.

To generate a convenient hematologic cell model for Fabry disease, peripheral blood was gathered from Fabry patients other than the skin fibroblast donor and from a normal volunteer donor under an approved National Institutes of Health protocol. Leukocytes were assayed for total α-gal and specific α-gal A enzyme activity as were B cell lines generated from patients with Fabry disease and a normal control generated after EBV transformation (Fig. 2 *A* and *B*). No major changes were seen in the specific enzyme activities indicating that the immortalization procedure did not significantly alter α-gal A enzyme production or stability. Patients 1 and 2 had

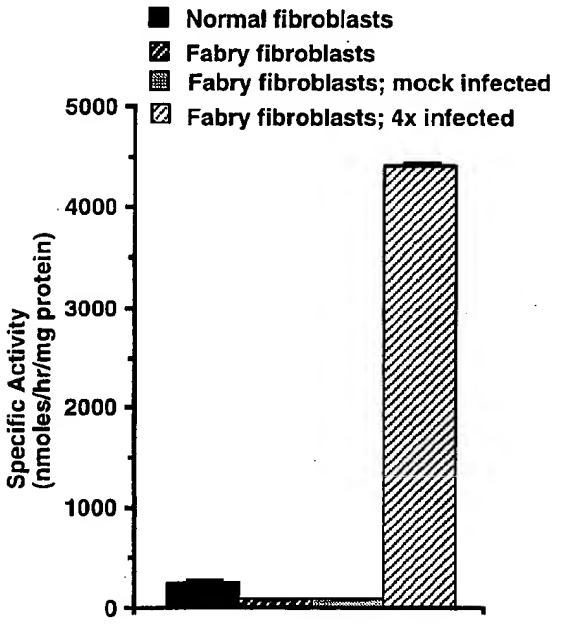

FIG. 1. Correction of the enzymatic defect in Fabry patient skin fibroblasts. Cells were infected 4× with supernatant from the AM12PG1α-gal A virus producer pool and assayed for enzyme activity. Bars represent α-gal A intracellular enzyme specific activity with standard error of the mean in all cases.

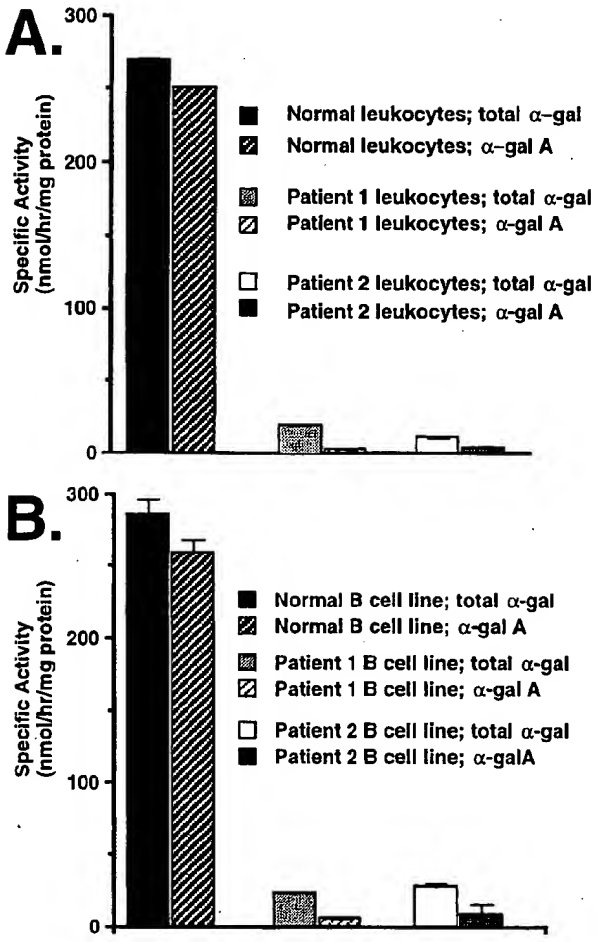

FIG. 2. Total α-gal and α-gal A intracellular enzyme activity levels in (*A*) patient peripheral blood leukocytes and in (*B*) patient immortalized B cell lines generated after EBV transformation.

total α-gal and α-gal A enzyme levels that were observed to be ≈1% of normal making these cells appropriate models for studies with recombinant transfer vectors or alternative DNA and enzyme delivery systems.

Significant increases in α-gal A activity in the patient B cell lines were observed after recombinant retroviral infection (Fig. 3). Correction to an enzyme level of 63% of normal was observed in one case, whereas correction to a level of greater than that seen in normal cells was observed in the other. Both of the corrected Fabry patient B cell lines showed greater than 100-fold increases in α-gal A activity levels. This finding is encouraging despite the fact that the infection of the patient B cell lines with the recombinant construct was likely quite inefficient (10), probably because of reduced amphotropic virus receptor present on these cells. In support of this concept, increasing the number of AM12PG1α-gal A infections of the patient B cell lines (up to 16×) increased the amount of intracellular enzyme produced in a correlative fashion (data not shown). This increased enzyme activity also correlated with increased proviral copy number as measured by comparative Southern blot analysis (data not shown). As a control to gauge the effects on total lysosomal compartment enzyme levels, β-hexosaminidase activity was largely unaffected under these conditions and manipulations (data not shown).

Secretion Studies. To determine whether extracellular secretion of α-gal A activity occurred in the corrected cells, culture media of the infected fibroblasts and the corrected B cells from Fabry patients were assayed for enzyme activity.

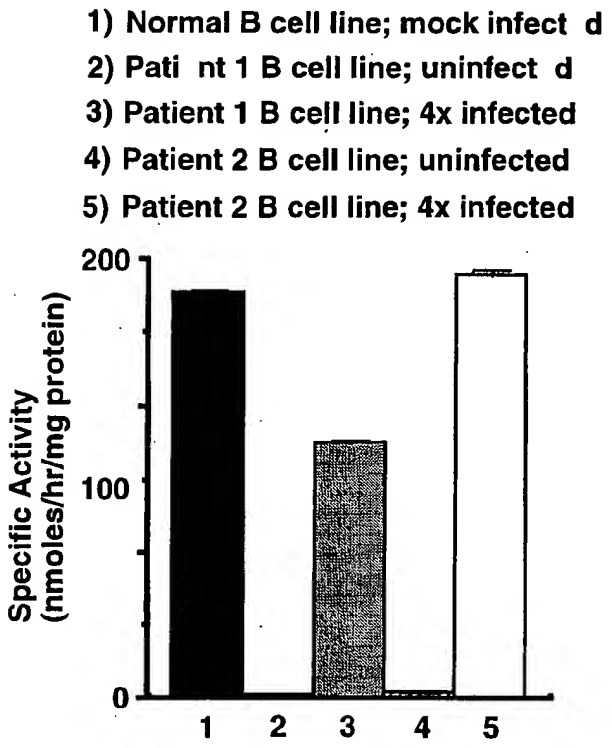

FIG. 3. Intracellular α-gal A enzyme specific activity levels obtained after infection of the the patient B cell lines with the recombinant retroviral supernatant from the AM12PG1α-gal A#8 virus producer line.

Media from normal, Fabry, and the Fabry mock-infected fibroblasts exhibited minimal enzyme activity (Fig. 4). In contrast, media from the pool of 4×-infected fibroblasts (representing about 2×10^6 cells) contained 8.4 nmol/hr/ml of media. This (10-fold) increase over background correlates well with the intracellular levels observed in Fig. 1.

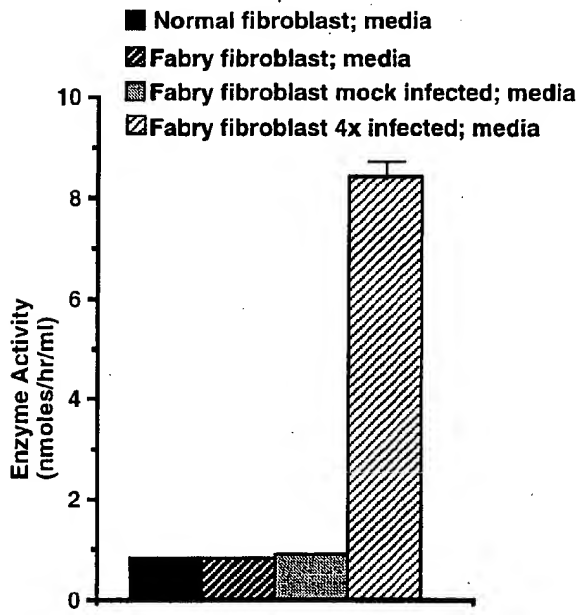

FIG. 4. Secreted α-gal A enzyme activity obtained from the infected patient fibroblasts.

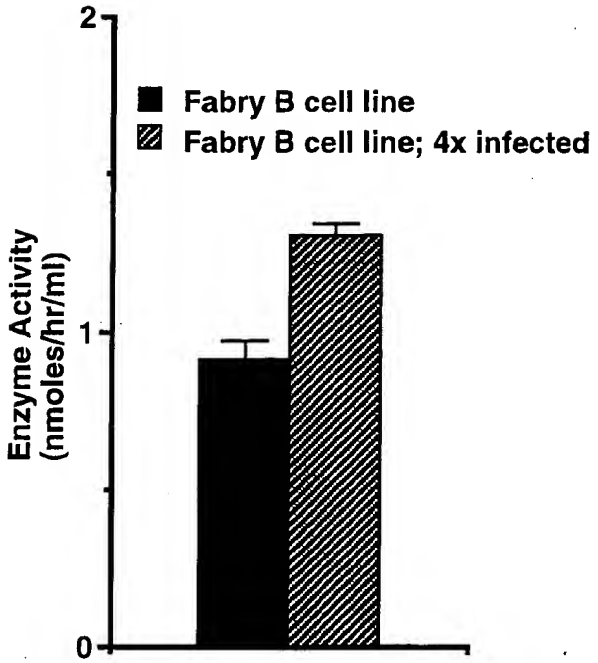

FIG. 5. Secreted α-gal A enzyme activity obtained from the infected Fabry patient B cell lines as a hematologic cell model.

A low, but significant ($P < 0.0001$ by t test; $n = 9$), amount of secreted α-gal A activity was observed in the media of the infected B cells grown at a density of $<10^6$ cells per ml (Fig. 5). The small amount of secreted enzyme from the infected B cells reflects the fact that the infection was likely less efficient than the fibroblasts. Differences in the lysosomal protein production and secretion pathways for each cell type may also play a role. As with the endogenous α-gal A activity, secreted activity increased linearly with increases in the transduction events of the patient B cell lines (data not shown).

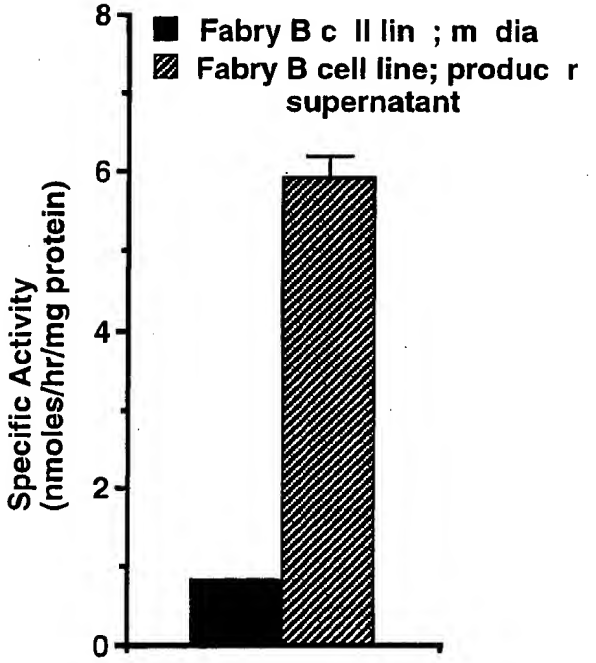

FIG. 7. Uptake of the α-gal A enzyme activity into the immortalized Fabry patient B cell line. Added enzyme activity was again derived from the multiply transduced virus-producer cell supernatant.

Uptake Studies. The uptake of the secreted enzyme by various uncorrected bystander cells was examined. Unconcentrated and unpurified media (specific to the cell type being assayed), obtained after incubation with the AM12PG1α-gal A#8 virus producer cells, was used as a source for these studies. When $\approx 2 \times 10^6$ skin fibroblasts, obtained from a patient with Fabry disease, were incubated with various amounts of the producer supernatant, increases >16-fold over background levels of enzyme were seen (Fig. 6). After mea-

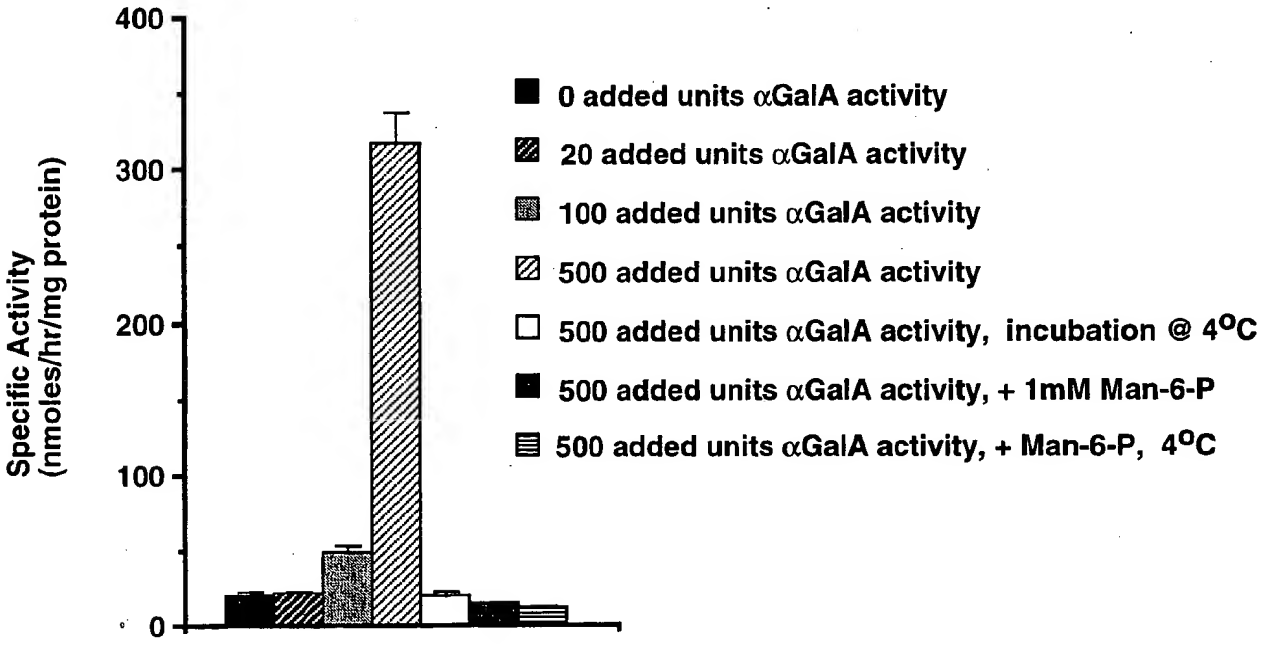

FIG. 6. Uptake of the α-gal A enzyme activity into uncorrected Fabry patient fibroblasts. Various units of secreted α-gal A activity from source supernatant were added to the cells and incubation, in the presence or absence of Man-6-P, was at 37°C unless noted.

surement of the total cell protein, it was established that about 6 units of enzyme were taken up in 3 hr out of the ≈500 units added. Additional studies revealed that this uptake was completely abrogated when cells and source supernatant were incubated at 4°C (to demonstrate specific uptake as opposed to extramembrane "sticking"). The uptake was nearly totally blocked when Man-6-P was added to the incubation indicating that the uptake was mediated through the Man-6-P receptor on these cells and was not due to a nonspecific import mechanism.

In the hematologic cell model, when $\approx 10^7$ uninfected patient B cells were incubated with 13 ml of the producer supernatant (representing about 1300 units of enzyme) and assayed for intracellular activity, more than a 7-fold increase of endogenous enzyme activity in the recipient cells was found (Fig. 7). Here it is estimated that 4 units of enzyme were taken up in 3 hr by these cells. Even at the lower per cell uptake of the soluble α-gal A for the Fabry B cell hematologic model ($\approx 10^3$ molecules per cell in 3 hr vs. $\approx 10^4$ molecules per cell for the Fabry fibroblasts), this finding further demonstrates that the metabolic cooperativity model is applicable to various types of bystander cells.

Uptake studies into uncorrected patient fibroblasts and the immortalized patient B cell models, were attempted with media collected from the 4×-transduced Fabry fibroblasts. No uptake, as measured by enzyme activity assays, above background was observed (data not shown). More sensitive detection procedures, i.e., radiolabeling of the added α-gal A protein, may be necessary to detect internalized enzyme in this case due to the relatively low amount of corrective enzyme secreted.

DISCUSSION

A recombinant retroviral vector has been constructed that engineers efficient transduction of cells and expression of human α-gal A activity. This vector is of high titer, and it corrects the enzymatic defect in multiple types of cells obtained from patients with Fabry disease. Further, the virus engineers expression of a form of α-gal A activity that is secreted and taken up, in a M-6-P receptor-specific manner, into unmodified bystander cells. Uptake of this secreted form of α-gal A was observed in fibroblasts and in hematologic cells derived from patients. Such transduced cells may provide a constant corrective function in trans. These findings represent the first observation of such a corrective effect for Fabry disease in these varied cell types.

Treatment for Fabry disease was initiated a number of years ago by infusion of normal human plasma (16) or partially purified preparations of α-gal A to patients (17, 18). The enzyme has a short half-life in circulation, however, and the corrective activity was rapidly cleared. A step toward the genetic correction of Fabry disease has recently been undertaken by the construction of a recombinant retroviral vector that delivers the α-gal A cDNA (19). Although efficient intracellular expression of the α-gal A was observed, the recombinant retrovirus produced was of low titer. This may have been due to the presence of the large insert in the vector added for the functional selection of corrected cells. In addition, no correction of patient cells was documented in that study.

Stable and marked overexpression of human α-gal A has been engineered in Chinese hamster ovary cells, and large amounts of secreted high-mannose forms of the enzyme were detected (20). This secretion was postulated to occur because of an aggregation of the overexpressed protein resulting in a reduced affinity for Man-6-P receptors in the trans Golgi network that directs protein to the lysosomes, causing some enzyme to be shunted to secretory pathways. It is possible that the overexpression observed in the present investigation is sufficient to cause a similar aggregation and secretion of α-gal A enzyme that has the requisite Man-6-P ligand for uptake by other cells. The lesser amount of secretion in the B cell model would then be consistent with the lower overall expression seen.

It is possible that corrected stem cells (and their progeny) from Fabry patients, after *ex vivo* transduction and reimplantation, may become a continual source of secreted α-gal A activity *in vivo*. This activity would then be circulating and able to be delivered and taken up by various target cell and tissue types. Corrected stem/progenitor cells may also have a growth advantage due to a reduction in their intracellular lipid load. If such an advantage is realized, it would increase the production of secreted enzyme and thereby increase the circulating amounts of protein available over a considerable period of time.

Metabolic cooperativity or "cross-correction" has been demonstrated in the context of other lysosomal storage disorders. Enzymatic cross-correction in mucopolysaccharidosis VII mice, derived from transduced stem/progenitor cells, reduced lysosomal storage in liver and spleen in this model (21). Effective correction of a mouse model for galactosialidosis was accomplished by transplantation of hematopoietic cells engineered to overexpress and secrete the corrective factor (22). Low but noticeable correction of the central nervous system from the overexpressing transplanted bone marrow was also observed in that study. These findings provide encouragement for the application of such correction to humans. Along these lines, lymphocytes from patients with Hunter syndrome were corrected endogenously for the enzymatic defect by retroviral-mediated gene transfer and cell-to-cell enzyme activity transmission occurred (23). Another recent study demonstrated sustained long-term expression and secretion of α-L-iduronidase, another lysosomal enzyme, in transduced human stem/progenitor cells (24). This further demonstrates the potential for metabolic cooperativity for many diseases of this type.

Studies where $CD34^+$ stem/progenitor cells from normal and Fabry patient donors are transduced are underway. These experiments are necessary to gauge the effectiveness of this viral construct in transducing these primitive repopulating cells and to establish whether long-term overexpression and secretion can be maintained in this system.

We thank Nick Flerlage (National Institute of Neurological Diseases and Stroke) for technical assistance, Dr. Gary Barsonian (Genzyme, Cambridge, MA) for the plasmid pGB78A, Dr. James Nagle (National Institute of Neurological Diseases and Stroke) for sequencing the retroviral plasmid construct, and Dr. Harry Malech (National Institutes of Health) for many helpful suggestions.

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L6 ANSWER 79 OF 94 CAPLUS COPYRIGHT 2003 ACS
AU Takenaka, Toshihiro; Hendrickson, Chad S.; Tworek, David M.; Tudor, Matthew; Schiffmann, Raphael; Brady, Roscoe O.; Medin, Jeffrey A.
TI Enzymatic and functional correction along with long-term enzyme secretion from transduced bone marrow hematopoietic stem/progenitor and stromal cells derived from patients with Fabry disease
SO Experimental Hematology (New York) (1999), 27(7), 1149-1159.

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L6 ANSWER 75 OF 94 MEDLINE DUPLICATE 31
AU Ohsugi K; Kobayashi K; Itoh K; Sakuraba H; Sakuragawa N
TI Enzymatic corrections for cells derived from Fabry disease patients by a recombinant adenovirus vector.
SO JOURNAL OF HUMAN GENETICS, (2000) 45 (1) 1-5.
Journal code: 9808008. ISSN: 1434-5161.

L6 ANSWER 76 OF 94 MEDLINE DUPLICATE 32
AU Takiyama N; Dunigan J T; Vallor M J; Kase R; Sakuraba H; Barranger J A
TI Retrovirus-mediated transfer of human alpha-galactosidase A gene to human CD34+ hematopoietic progenitor cells.
SO HUMAN GENE THERAPY, (1999 Dec 10) 10 (18) 2881-9.
Journal code: 9008950. ISSN: 1043-0342.

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Experimental Hematology 27 (1999) 1149–1159

July

Enzymatic and functional correction along with long-term enzyme secretion from transduced bone marrow hematopoietic stem/progenitor and stromal cells derived from patients with Fabry disease

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(Received 23 November 1998; revised 29 January 1999; accepted 26 February 1999)

Fabry disease is a lysosomal storage disorder that is due to a deficiency in α-galactosidase A (α-gal A). Previously we have shown that a recombinant retrovirus synthesized for the transfer of the human α-gal A coding sequence was able to engineer enzymatic correction of the hydrolase deficiency in fibroblasts and lymphoblasts from Fabry patients. The corrected cells secreted α-gal A that was taken up and utilized by uncorrected bystander cells, thus demonstrating metabolic cooperativity. In separate experiments we used transduced murine bone marrow cells and successfully tested and quantitated this phenomenon in vivo. In the present studies, which were designed to bring this therapeutic approach closer to clinical utility, we establish that cells originating from the bone marrow of numerous Fabry patients and normal volunteers can be effectively transduced and that these target cells demonstrate metabolic cooperativity. Both isolated CD34$^+$-enriched cells and long-term bone marrow culture cells, including nonadherent hematopoietic cells and adherent stromal cells, were transduced. The transferred gene generates increased intracellular α-gal A enzyme activity in these cells. Further, it causes functional correction of lipid accumulation and provides for long-term α-gal A secretion. Collectively, these results indicate that a multifaceted gene transfer approach to bone marrow cells may be of therapeutic benefit for patients with Fabry disease.

Keywords: **Fabry disease—Retroviral gene transfer—CD34$^+$ cells—Long-term bone marrow culture**

Introduction

Fabry disease is caused by an in-born genetic defect of the lysosomal enzyme α-galactosidase A (α-gal A) [1]. Clinical manifestations in patients with this X-chromosome–linked disorder are due to an accumulation in cells of glycosphingolipids with terminal α-galactosyl residues. These effects, resulting from a wide variety of mutations in the α-gal A gene [2], are evident in a number of organs and tissues but are especially pernicious in the renal, cardio- and cerebrovasculature [3]. There is currently no cure for this disorder, and patients have a poor prognosis.

Bone marrow, and to a lesser extent the liver, is postulated to be a primary source of the problematic lipids in Fabry disease [4]. This is due to the synthesis of glycosphingolipids in cells of that compartment and their subsequent incorporation into differentiating and maturing blood components [4]. High levels of lipids with terminal galactosyl residues also occur in the plasma of Fabry patients [5]. These lipids are likely to be partly derived from intracellular synthesis, but most appear to be derived from the incomplete catabolism of cellular membrane components of senescent erythrocytes by macrophages [4]. The circulating glycosphingolipid is then complexed with serum lipoproteins [6] and together taken up by vascular endothelial and smooth muscle cells with prolonged accumulation of this nature contributing to some of the clinical manifestations of this disorder.

Transplantation of bone marrow cells as therapy for Fabry disease has not been described, although in the 1970s transplantation of hematopoietic stem cells obtained from fetal livers was reported to improve the clinical course of the disorder in three patients [7]. A small, distinct subset of hematopoietic cells, the CD34$^+$ population, contains precursors to all blood cell lineages and can completely reconstitute hematopoiesis after bone marrow transplantation [8]. This population of cells has been a prime target for corrective gene transfer for disorders that have hematopoietic components [9–11]. The CD34$^+$ population of cells also includes vascular endothelial cell progenitors [12,13], cells that are pivotal to Fabry disease treatment strategies. As

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0301-472X/99 $–see front matter.
PII S0301-472X(99)00050-8

with many other lysosomal storage disorders, it may be possible then that correction for Fabry disease may be effected by transfer of the correct cDNA coding sequence into these cells. Toward this goal, recombinant retroviruses have recently been constructed [14,15]. In the latter study [15], enzymatic correction in cells derived from patients with Fabry disease occurred. Secreted α-gal A activity was observed from transduced cells that was incorporated, in a mannose 6-phosphate–dependent manner, into uncorrected bystander cells. Furthermore, we have recently created a system to examine and quantitate this phenomenon in vivo (Takenaka et al., submitted). In that study, we generated a recombinant retrovirus encoding a fusion form of α-gal A with a specific and unique tag at the carboxy terminus. We showed that circulating specific enzyme could be produced by transduced and transplanted bone marrow cells and that these effects, including engraftment of marked cells, were stable over time.

These concepts lead to the premise that corrective enzyme for treatment of patients with Fabry disease could be delivered to important target cells throughout the body by transduction of appropriate cells of the hematopoietic system. Further, gene correction of myeloid progenitors of the bone marrow of Fabry patients could produce offspring macrophages that are enabled to completely degrade lipid components of senescent erythrocyte membranes. Correction of bone marrow-derived cells themselves has the potential to reduce lipid load at a point of origin, as well as then to deliver beneficial amounts of enzyme through the observed secretion effects into the circulation that could be utilized elsewhere. Modulation of this "cross-correction" effect may eventually overcome the poor marking efficiency and low therapeutic efficacy that has been observed in other retroviral transduction clinical protocols.

The present preclinical study demonstrates that transduction of bone marrow cells has merit as a potential treatment for Fabry disease. Cells derived from the bone marrow of multiple patients with Fabry disease are transduced at an appreciable rate. These cells are fully corrected for the enzymatic deficiency; they are also functionally corrected, and secretion of α-gal A occurs over extended periods of time.

Materials and methods

Cell culture and virus production

Cells were grown at 37°C in the presence of 5% carbon dioxide. The AM12/PG1a-galA#8 recombinant amphotropic retroviral vector producer cell line [15] was used for all transduction experiments. Marker rescue assays using lengthy potential amplification periods, multiple dilutions, and dual complete assay repeats were performed as described [16] on this producer line to determine if replication competent virus (RCR) were present. No RCR was found at any supernatant dilution.

Stromal cell layer preparation

Bone marrow specimens were obtained with informed consent under an approved NIH protocol. A bone marrow aspirate from a normal volunteer was used to prepare stromal cells for all transductions of $CD34^+$-enriched cells that used this support. For this, mononuclear cells were collected using lymphocyte separation medium (LSM; Organon Teknika Cappel, Durham, NC), washed with Dulbecco's modified Eagle medium (DMEM; Life Technologies, Gaithersburg, MD) plus 2% fetal bovine serum (FBS; Life Technologies), and resuspended in DMEM with penicillin/streptomycin/glutamine (pen/strep/gln; Life Technologies) containing 15% FBS and 0.25 mg/mL Fungizone (Life Technologies). The suspended cells were seeded into 175-cm^2 flasks (Costar Corp., Cambridge, MA). In the transduction experiments, a confluent layer of normal stromal cells was gamma irradiated (~1100 rad) prior to addition of patient and normal $CD34^+$-enriched cells and transduction components.

Isolation and transduction of human bone marrow $CD34^+$-enriched cells

$CD34^+$ cells were enriched from Fabry patients (Fabry #1, #2, #3, and #4) and normal volunteer (Normal #1, #2, and #3) bone marrow aspirates in the following manner: After centrifugal enrichment for mononulear cells (prepared as described earlier), the Celprate LC system (Cell Pro, Inc., Bothell, WA) was used to isolate cells expressing the CD34 cell surface marker. In seven different $CD34^+$ preparations of Fabry patient and normal cells starting with 38 to 48 mL of bone marrow aspirates, 6×10^7 to 3×10^8 mononuclear cells yielded 4×10^5 to 3×10^6 cells after the $CD34^+$ enrichment. Enrichment using this procedure has been shown to give purities of around 70% $CD34^+$ cells [17,18]. In one case (Normal #2) a different enrichment procedure was used. The StemSep lineage depletion kit (StemCell Technologies, Inc., Vancouver, Canada) was used according to the manufacturer's instructions. This depletion procedure has been shown to give a $CD34^+$ cell purity of about 70%.

Approximately 16 hours prior to transductions, the media on the viral producer cells was changed to that used to grow the human bone marrow cells (DMEM with 15% FBS and pen/strep/gln) to allow viral accumulation in a supportive media context. Transductions of $CD34^+$ cells were performed as described [16] with some modifications. Briefly, the $CD34^+$-enriched cells were transduced four times with filtered viral supernatant in the presence of stromal cells, 4 μg/mL of protamine sulfate (Elkins-Sin, Cherry Hill, NJ), 50 ng/mL of recombinant human IL-6 (R & D Systems, Minneapolis, MN), and 100 ng/mL of recombinant human stem cell factor (R & D Systems). Mock transductions were performed by using naive AM12 cell supernatant on stromal support with protamine sulfate and growth factors.

For some experiments, slightly different transduction conditions were used. In one series (results listed in Table 1), cells were transduced on stroma support as described earlier and then transferred to a new flask without stroma for 24 hours to decrease the likelihood of the normal cells contributing to the enzyme assay results by allowing reattachment of any transferred stromal cells prior to collection of nonadherent cells for the assay. In another series of experiments, to avoid the potential contribution of transduced stromal cells in experiments designed to observe secreted enzyme activity from $CD34^+$ cells themselves, the enriched stem/progenitor cells were transduced four times by the addition of producer supernatant with supplements only. No difference in transduction efficiency, as measured by polymerase chain reaction (PCR) analysis, was found for the transductions without stromal support (data not shown).

Table 1. Intracellular lysosomal enzyme activities in control and transduced Fabry patient and normal $CD34^+$-enriched cells

Cell source		Enzyme activity (nmoles/hour/10^6 cells)		PCR results
		α-Galactosidase A	β-Hexosaminidase	
Fabry patient #1	Mock	0.32 ± 0.03	ND	
	4×-transduced	10.2 ± 0.04	ND	12/34 = 35% positive
Fabry patient #2	Mock	0.15 ± 0.00	3589 ± 2009	
	4×-transduced	3.01 ± 1.93	3059 ± 1545	7/36 = 19% positive
Fabry patient #3	Mock	0.06 ± 0.01	490 ± 6	
	4×-transduced	1.85 ± 0.03	557 ± 12	9/28 = 26% positive
Normal #1	Untouched	8.62 ± 0.54	669 ± 8	
	Mock	9.56 ± 0.15	605 ± 51	
Normal #2	Mock	25.3 ± 1.1	ND	
	4×-transduced	32.0 ± 2.6	ND	31/76 = 41/% positive

The cells were sequestered from bone marrow aspirations by immunoaffinity enrichment, transduced four times with recombinant retroviral supernatant (see Materials and methods), plated for 24 hours (to allow attachment of any possible irradiated stromal cells that may have been transferred), and then subjected to enzyme assays on extracts of the nonadherent cells. Mock-transduced cells were maintained identically except that naive AM12 cell supernatants were substituted for recombinant vector supernatant in the transduction protocol. Also included in the table are the results of the PCR analyses for the presence of the recombinant proviral DNA band as assayed on plated and collected hematopoietic progenitor cell colonies derived from transduced cell samples.

ND = not determined.

After transductions, the cells were assayed for enzyme activity or plated in methylcellulose semisolid support medium (MethoCult GF H4434 or H4435, StemCell Technologies) to allow differentiation and colony formation.

Estimation of transduction efficiency

Methylcellulose cultures of cells were allowed to grow until macroscopic progenitor colonies had formed. The colony types were identified visually by morphology and well-isolated colonies were collected as described [19] for a PCR-based analysis of the efficiency of transduction of progenitor cells. Either 5 or 10 μL of the progenitor DNA solution was used as a template for 50-μL PCR reactions containing 2.5 U AmpliTaq DNA polymerase (Roche, Branchburg, NJ), 5 μL Perkin Elmer Taq II buffer (Roche), 1.25 mM $MgCl_2$ (Roche), 200 μM dNTPs (Life Technologies); 264 pM DNA oligonucleotide primers (Bio-Synthesis Inc., Lewisville, TX), 5 μL PCR dye buffer (60% sucrose; Sigma, St. Louis, MO), 1 mM cresol red; Sigma), and water to volume. PCR primers were designed to bind in exons 6 and 7 of the human α-gal A gene, spanning an intron to allow discrimination of the genomic and proviral copies of the gene. The sequences of the primers were 5′-CGACACATCAGCCCTCAAGC-3′ and 5′-TTAAAGTAAGTC-TTTTAATGACATC-3′. Optimal PCR conditions were empirically determined to be 95°C for 3 minutes, 53°C for 1 minute, and 72°C for 1 minute for one cycle followed by 95°C for 1 minute, 53°C for 1 min, 72°C for 1 minute for 25 cycles followed by 95°C for 1 minute, 53°C for 1 minute, and 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis and blotted to a nylon membrane (Hybond-N; Amersham Life Science, Buckinghamshire, United Kingdom). Specific products were detected by probing with a biotinylated fragment from a ScaI/KpnI digest of the α-gal A cDNA and detected using an avidin/alkaline phosphatase conjugate with a CSPD chemiluminescent substrate (random-primed biotin labeling kit and Southern detection kit; Tropix, Inc., Bedford, MA). The efficiency of transduction into progenitor cells was estimated by dividing the number of reactions showing both genomic and positive cDNA bands by the total of those showing strong genomic bands. Only reactions in which the intensity of the proviral band was as strong as approximately one-half of the intensity of the genomic band were scored as positive to minimize the possible inclusions of false positives.

Human long-term bone marrow cultures and transductions

Long-term bone marrow cultures (LTBMC) were initiated from bone marrow aspirates of patients with Fabry disease (Fabry #5 and #6) and a normal volunteer (Normal #4) by seeding isolated mononuclear cells into 25-cm^2 flasks using MyeloCult H5100 medium (StemCell Technologies) supplemented with 1 μM hydrocortisone (Sigma) and pen/strep/gln with 0.5 μg/mL Fungizone. Approximately 10^8 cells were seeded into flasks and maintained as described [20] in a total volume of 10 mL. The cultures were initiated at 37°C and then transferred after 3 days to 33°C. Each week thereafter, the nonadherent cells were demipopulated by a gentle rocking of the flask. At this point, one-half of the media was replaced with either fresh medium (for untouched samples) or fresh medium conditioned on viral producer cells for 24 hours (to collect retroviral supernatant containing packaged virus). Mock-transduced cells were incubated with media conditioned on wild-type AM12 cells only. Collected cells and media were then assayed for α-gal A activity (see following). Two distinct schedules of transduction were used for Fabry patient and normal control LTBMC cells to compare efficacy. Schedule 1 [21] utilized three once weekly transductions (three total), beginning with the second day of LTBMC, occurring with weekly media changes that allow stimulation of cell division and possible enhancement of transduction. For schedule 1, one-half of the supernatant was discarded and replaced with an equal volume of filtered overnight retroviral vector-containing or mock supernatant. Schedule 2 [22] consisted of four once daily infections (four total) starting on the first day of culture. This was done by removal of one-half of the LTBMC media, recovery of cells by centrifugation, resuspension in overnight retroviral vector supernatant made with the LTBMC medium, and readministration back into the original flasks. After various weeks of LTBMC, cells and media were collected for enzyme assay and

nonadherent cells were plated in progenitor colony assays for estimations of transduction as measured by PCR analyses.

Western blot analysis

Western blot analysis was performed to detect actual expression of α-gal A in transduced bone marrow cells derived from a patient with Fabry disease (Fabry #7). Cells from a normal volunteer (Normal #5) were used as control. For this Western blot, 7 μg of protein from cell extracts was separated by 15% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane (Hybond-P; Amersham). The membrane was incubated with rabbit anti-human α-gal A polyclonal antibody [23] followed by peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH). Specific binding was detected by an enhanced chemiluminescence procedure (ECL Plus; Amersham).

Immunofluorescence staining of ceremidetrihexoside

Immunofluorescence staining of ceremidetrihexoside (CTH) was performed according to the method described previously [24] with some modifications. Cells that were seeded and grown on cover slips were fixed with 2% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS), pH 7.4 (Life Technologies) for 30 minutes on ice. After removal of the fixing solution, the specimens were incubated with 5% (w/v) bovine serum albumin (Fraction V; ICN Biomedicals, Inc., Aurora, OH) in PBS for 1 hour at room temperature for blocking. The specimens were next incubated overnight at 4°C with monoclonal mouse anti-CTH IgG [25] (kindly supplied by Dr. Hitoshi Sakuraba, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and then washed with ice-cold PBS. Specific binding was visualized with fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse IgG (Cappel) on an Axioplan fluorescent microscope (Zeiss, Germany) and photographed.

Enzyme assays

α-gal A enzyme activity was measured in each sample. B-Hexosaminidase enzyme activity in each sample was measured as a control. The assays were modified from those previously described [15,23]. Briefly, nonadherent cells from flasks containing LTBMC or $CD34^+$ cells on stromal support were gently collected, washed with PBS, and counted with a hemocytometer. Media, after clarification by centrifugation and filtration through a-0.45μm syringe filter (Millipore, Bedford, MA), also was assayed. To assay α-gal A activity in progenitor colonies, cells were collected from methylcellulose plates by gentle scraping and combined with PBS in 15-mL centrifuge tubes (Sarstedt Inc., Newton, NC). Cells were collected by low-speed centrifugation (~300g) and washed thoroughly in PBS. The cell pellets were resuspended in 220 μL of assay buffer (28 mM citric acid/44 mM disodium phosphate, pH 4.4; Sigma) with 3 mg/mL sodium taurocholate (Sigma). After sonication for 5 seconds, the lysates were cleared by centrifugation at 14,000 rpm for 30 minutes in a refrigerated microcentrifuge. Various amounts of cleared lysates and media were used in enzyme assays. The volumes were adjusted to 200 μL with filter-sterilized substrate solutions (for α-gal A: 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (Research Products International [RPI], Mt. Prospect, IL) with 100 mM N-acetyl-D-galactosamine (Sigma) as an inhibitor of α-N-acetylgalactosaminidase in assay buffer; for β-hexosaminidase: 5 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide [RPI] in assay buffer) and incubated for various times. Assays for blanks were performed simultaneously for each α-gal A and β-hexosaminidase assay point. For this assay, substrate was added but no enzyme source was included in the incubation to measure autoconversion of the fluorogenic substrate. The fluorometer values obtained for the blank were subtracted from the fluorometer values obtained for each enzyme assay point. A minimum of three samples were assayed for each data point. Results are reported as mean activity ± SEM obtained for each assay. Protein was measured by standard Lowry assay techniques. α-gal A activity was normalized to the number of cells or protein concentration.

Results

Enzymatic correction in $CD34^+$-enriched and progenitor colony cells derived from patients with Fabry disease

Fabry patient and normal volunteer $CD34^+$ cells were enriched from bone marrow aspirates and transduced. Three distinct bone marrow collections and transductions were performed on different Fabry patient (Fabry #1, #2, and #3) bone marrow specimens. Table 1 shows the α-gal A activity obtained in the recombinant virus-transduced $CD34^+$-enriched cells maintained in culture compared with mock-transduced Fabry patient cells and normal control (Normal #1 and #2) cells. Mock-transduced cells from patients with Fabry disease demonstrated an α-gal A activity ranging from 0.06 to 0.32 nmoles/hour/10^6 cells that increased to 1 to 3 nmoles/hour/10^6 cells following transduction. This is a significant increase from 3- to 30-fold, depending on the sample, although the absolute levels of enzyme activity never reached that seen in these normal specimens. Normalization of enzyme activity was done to cell numbers to allow comparison with earlier studies and because the total amount of cell protein recovered was low. β-Hexosaminidase activities were found to be similar in the transduced cells and mock-transduced cells, although wide variations were seen between different patient cells and some variation occurred even in samples from the same donor (Table 1). These results are the first reports of intracellular α-gal A enzyme levels in this $CD34^+$-enriched cell population. The level of α-gal A activity in the mock-transduced Fabry patient $CD34^+$-enriched cells is comparable to that of unfractionated leukocytes obtained from hemizygous patients with Fabry disease [26]. With the addition of the recombinant retrovirus, the α-gal A activity in the transduced $CD34^+$-enriched cells became comparable to that seen in leukocytes from obligate heterozygotes and even approached the level seen in some normal cells [26].

A PCR-based assay was developed to appraise the efficiency of gene transfer into progenitor cells derived from human bone marrow using the AM12/PG1a-galA#8 [15] producer cell supernatant. Primers, PCR conditions, and probes were empirically selected to minimize cross-reactivity and ambiguity with genomic sequence in analyses. The progenitor colonies were identified by morphology and collected (see Methods). Clearly distinguishable and specific proviral (389 bp) and genomic (645 bp) bands were ob-

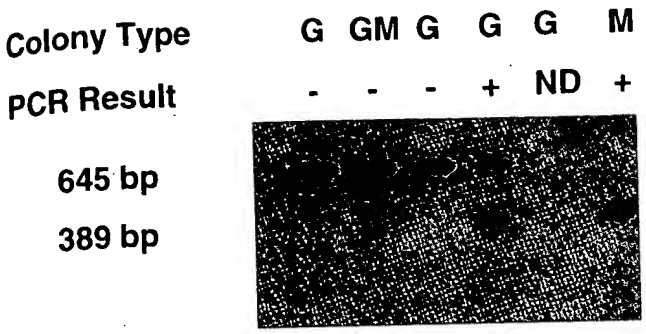

Figure 1. Representative PCR assay for the detection of proviral DNA sequences in various colonies identified by morphology and isolated from methylcellulose semisolid support. Colony identity: G = granulocyte; GM = granulocyte-macrophage; M = macrophage; ND = not determinable. The genomic band is 645 bp and the proviral band is 389 bp.

tained after the PCR and probing techniques (Fig. 1). In addition, no gross differences were observed in colony numbers from the control and transduced normal and Fabry patient cells (data not shown). Table 1 shows the pooled results obtained from these PCR analyses testing for the presence of the proviral DNA in plated progenitor colonies derived from each population of transduced $CD34^+$-enriched cells. Conservative transduction efficiencies into progenitor cells with this vector and transduction protocol ranged from 19-41%. It should be noted that the PCR data obtained are for rough comparisons of different transductions only and are not likely to reflect the marking rate of true repopulating hematopoietic stem cells.

Cells were collected and assayed for enzymatic activity from the progenitor colonies. Table 2 shows the α-gal A activity (after normalization to number of cells) obtained from the transduced and mock-transduced Fabry patient (Fabry #1 and #3) colony cells along with the activity obtained from control normal (Normal #1 and #2) cells. A more than twofold increase in α-gal A specific activity in the retrovirally transduced cells was observed in one Fabry patient cell specimen, whereas a greater than sevenfold increase in enzyme activity was seen in the other. β-Hexosaminidase activities were found to be similar in the transduced and mock-transduced cells (Table 2). A high level of α-gal A activity was documented in all sets of normal cells that did not occur in colonies derived from the transduced or mock-transduced Fabry cells.

Enriched $CD34^+$ cell populations from a patient with Fabry disease (Fabry #4) and normal volunteers (Normal #2 and #3) were transduced and examined themselves for enzyme secretion. These transductions were done with supernatant and growth factors only (without stroma) to evaluate the effect of the isolated cells themselves. The transduced and control mock-transduced cells were washed well and then incubated in growth media and cytokines only for an additional 24 hours following the transduction protocol to minimize the possible contribution of α-gal A activity resident in the added retroviral producer supernatant to the secretion effect. The α-gal A activities in the media from mock-transduced and transduced Fabry #4 cells were 0.14 ± 0.01 and 0.19 ± 0.04 nmol/hour/mL, and statistical significance was detected by a paired *t*-test between these numbers. The α-gal A activities in the media from mock-transduced and transduced Normal #2 cells were 0.27 ± 0.00 and 0.54 ± 0.04 nmol/hour/mL, whereas the media enzyme activities from mock-transduced and transduced Normal #3 cells were 0.06 ± 0.03 and 0.19 ± 0.03 nmol/hour/mL, respectively. Cells transduced with the recombinant retrovirus showed very low but significant levels of secretion over control for each analysis. The β-hexosaminidase activities in the media from mock-transduced and transduced Fabry #4 cells were 152 ± 0 and 149 ± 2 nmol/hour/mL. The β-hexosaminidase activities in the media from mock-transduced and transduced Normal #2 cells were found to be 813 ± 7 and 800 ± 5 nmol/hour/mL, whereas the enzyme activity in the media from mock-transduced and transduced Normal #3 cells were 883 ± 45 and 899 ± 35 nmol/hour/mL, respectively. PCR analyses of progenitor colonies derived from the transduced normal cells showed 26% and 41% of colonies to be positive for the proviral DNA band. No concentration of the media occured for the assays and the cells were maintained at a low density of 1 to 2 × 10^5 cells/mL in all cases. The overall levels of secreted α-gal A activity in the media were much lower than seen previously for transduced fibroblasts and for transduced lymphoblasts [15], although the total cell numbers in the media in those cases were higher and transduced cell percentage has not been factored into the comparison.

LTBMC: correction and secretion

Using a long-term method to examine transduction efficiencies, cellular correction, and enzyme secretion effects, LTB-

Table 2. Intracellular lysosomal enzyme activities in collected Fabry patient and normal hematopoietic progenitor colony cells

		Enzyme activity (nmoles/hour/10^6 cells)	
Cell source		α-Galactosidase A	β-Hexosaminidase
Fabry patient #1	Mock	0.10 ± 0.00	695 ± 13
(from Table 1)	4×-transduced	0.24 ± 0.06	681 ± 8
Fabry patient #3	Mock	0.02 ± 0.00	632 ± 10
(from Table 1)	4×-transduced	0.14 ± 0.00	675 ± 48
Normal #1	Untouched	17.1 ± 0.4	977 ± 41
(from Table 1)	Mock	15.1 ± 1.1	709 ± 22
Normal #2	Mock	52.8 ± 1.2	2134 ± 47
(from Table 1)	4×-transduced	57.9 ± 2.5	2276 ± 19

The cells were transduced as described (see Materials and methods) and plated into methylcellulose support 24 hours after the last transduction procedure. Cells were allowed to expand into colonies for 10 to 14 days and then collected and extracts made for enzyme assays. All of the samples are derived from the same $CD34^+$-enriched and transduced samples as described in Table 1.

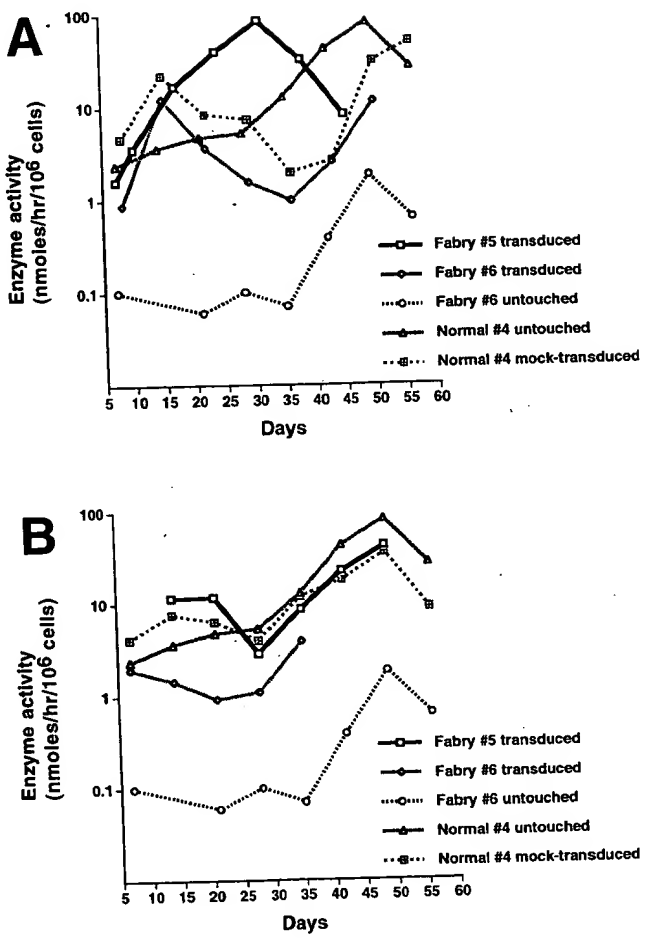

Figure 2. Intracellular α-gal A activities measured on various days from nonadherent cells collected from long-term bone marrow cultures (LTBMC). Data from untouched and transduced cultures are shown for two separate Fabry samples and a normal control. Each data point (also in Fig. 3) represents the mean of triplicate (or more) enzyme assays. Standard error bars for each enzyme assay point were plotted but are too low to be observed on this graph. **(A)** Cells transduced with schedule 1 (one time per week for 3 weeks = three total transductions). **(B)** Cells transduced with schedule 2 (four transductions at the onset of LTBMC = four total transductions). The Fabry patient #6 culture, transduced with this schedule, was stopped early due to contamination.

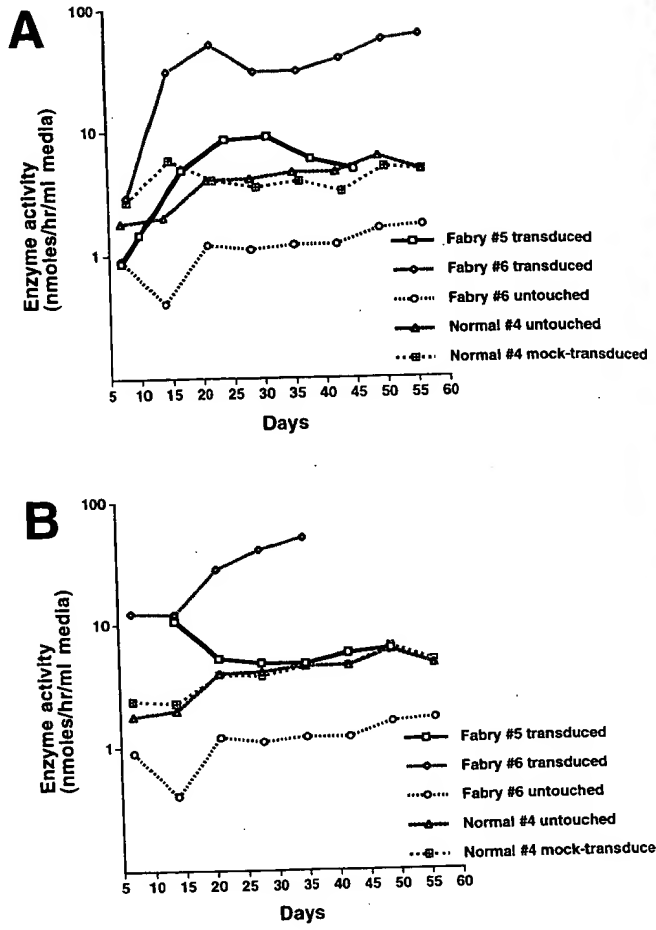

Figure 3. Secreted α-gal A activity at various days obtained directly from the long-term bone marrow culture (LTBMC) media without any manipulations. **(A)** LTBMC transduced with schedule 1 (one time per week for 3 weeks = three total transductions). **(B)** LTBMC transduced with schedule 2 (four transductions at the onset of LTBMC = four total transductions).

MCs were initiated from Fabry patient (Fabry #5 and #6) and normal (Normal #4) bone marrow mononuclear cells and transduced with recombinant virus. The growth characteristics of the cultures were consistent as similar numbers of adherent cells were recovered at the end of the culture periods for both the Fabry patient and normal cells. This includes both transduction schedules and the mock-transduced cultures (data not shown). Enzyme activities and transduction efficiencies were obtained on nonadherent cells on a number of days by fluorometric enzyme assay and by PCR analysis on plated progenitors.

Figure 2 shows the results of the intracellular α-gal A activity assay on nonadherent Fabry patient cells obtained at various time points after the initiation and transduction of the LTBMCs. Mock-transduced and untouched normal cells were assayed and compared as controls. Figure 2A shows the results obtained from transduction of two separate Fabry patient samples with transduction schedule 1. Figure 2B shows the results of transduction when schedule 2 was followed. In both cases, large increases in enzyme activity were seen even at the first data point measured (~1 week). This increase was observed throughout the culture period; enzyme levels were found to approximate normal values for both transduction schedules. Somewhat higher overall levels of enzyme activity were seen in the samples where transduction schedule 1 was used, although a large amount of variance from week to week was present, whereas cells transduced with schedule 2 seemed to have a later peak of enzyme activity.

Figure 3 shows the results of the α-gal A activity present in the media of the LTBMCs. No concentration of the media or any other enrichment for activity was performed. Again, part A demonstrates the enzyme activity data obtained using

transduction schedule 1 and part B values are obtained following transduction schedule 2. In both cases, large increases in the enzyme activity in the media were seen that were stable over time. In fact, for both transduction schedules, the secreted enzyme activity was found to be greater from the transduced Fabry patient cells than that observed from normal cells. This finding effectively extends the previous observations that transduced cells secrete α-gal A activity [15] to include transduced cells originating from bone marrow. Little variance in the media enzyme activity was seen for any of the untouched and mock-transduced cells from both normal volunteers and Fabry patients. This result indicates that the secreted α-gal A levels from normal cells is fairly low and that retroviral-mediated transduction of patient cells can increase the levels of circulating enzyme significantly.

PCR analyses for the presence of the proviral DNA band were performed at various times on progenitor colonies derived from the nonadherent cells of the transduced Fabry patient LTBMC samples (as shown in Fig. 1). The percentages of PCR positive colonies in comparison to the total number analyzed mirror the transduction frequencies shown for the $CD34^+$-derived colonies and transductions (data not shown). With this viral supernatant and the differing transduction protocols, the transduction efficiency of progenitor colonies was consistent at approximately 25% of the assayed colonies for all tests. There were some slight differences depending on the transduction schedule used, as schedule 1 seemed to generate slightly higher transduction frequencies, especially in Fabry transduced #2 (data not shown).

Relative contribution of adherent and nonadherent cells to the observed LTBMC secretion effects and detection of expressed α-gal A in transduced adherent cells

Experiments were performed to determine the bulk of the source of the secreted α-gal A enzyme activity in the LTBMC. Parallel cultures were initiated from independent Fabry patient (Fabry #7) and normal (Normal #5) bone marrow samples. The cells were transduced according to schedule 2 and maintained for 2 weeks. At this point, both cultures were depleted of nonadherent cells and media. One culture (culture 1) then received nonadherent cells returned with media and the other culture (culture 2) received an identical amount of media alone. Both sets of cultures were incubated for 24 hours and then the media was assayed for α-gal A activity. The results are shown in Table 3. Very slight (if any) increases in the media α-gal A enzyme activity from the LTBMC are seen when the nonadherent cells are added back to the culture, indicating that the adherent cells are providing most of the soluble enzyme. The nonadherent cells themselves were assayed for α-gal A enzyme activity and showed up to fourfold higher levels (data not shown), which is consistent with earlier results and indicates a productive transduction. To further explore the utility of the adherent cells as an enzyme source, the attached cells from these cultures were expanded in culture for an additional 2 weeks. At this point the adherent cells (only) were collected and assayed. Both Fabry patient transduced samples showed greater than tenfold higher α-gal A activity than the mock-transduced controls (see following).

To detect the actual expression of α-gal A in transduced bone marrow cells, a Western blot analysis was performed using transduced Fabry (Fabry #7) adherent cells. Mock-transduced Fabry (Fabry #7) adherent cells and mock-transduced normal (Normal #5) adherent cells were used as controls. These cells were cultured for 4 weeks and harvested for assay. In culture 1, α-gal A activities in transduced Fabry adherent cells, mock-transduced Fabry adherent cells, and mock-transduced normal adherent cells were 360.0 ± 8.9, 28.7 ± 1.3, and 1655.9 ± 34.1 nmol/hour/mg protein, respectively. In culture 2, α-gal A activities in transduced Fabry adherent cells, mock-transduced Fabry adherent cells, and mock-transduced normal adherent cells were 338.9 ± 10.2, 24.7 ± 0.4, and 1535 ± 31.5 nmol/hour/mg protein, respectively. By Western blot analysis, using an anti-human α-gal A polyclonal antibody, a band corresponding to the α-gal A protein was specifically detected not only in mock-transduced normal cells but also in transduced Fabry patient cells (Fig. 4), whereas no detectable band was observed in the

Table 3. α-Galactodisase A activities in long-term bone marrow culture media with and without nonadherent cells

Cell source		Enzyme activity (nmoles/hour/mL)	
		Culture 1 (+) nonadherent cells	Culture 2 (−) nonadherent cells
Fabry patient #7	Mock	1.66 ± 0.07	1.66 ± 0.00
	4×-transduced	4.33 ± 0.10	4.28 ± 0.05
Normal #5	Mock	7.65 ± 0.12	6.91 ± 0.43
	4×-transduced	12.07 ± 0.33	10.84 ± 0.23

Bone marrow was collected and transduced according to schedule 2 (see Materials and methods) and then maintained in parallel samples in culture for 2 weeks. The nonadherent cells were then vigorously removed from both cultures, and the cultures were washed to remove any remaining nonadherent cells. Subsequently, one set of adherent cells was returned with media to one of the parallel cultures. The other culture received long-term bone marrow culture media alone. The cultures then were incubated for 24 hours. The media was collected, clarified, and assayed.

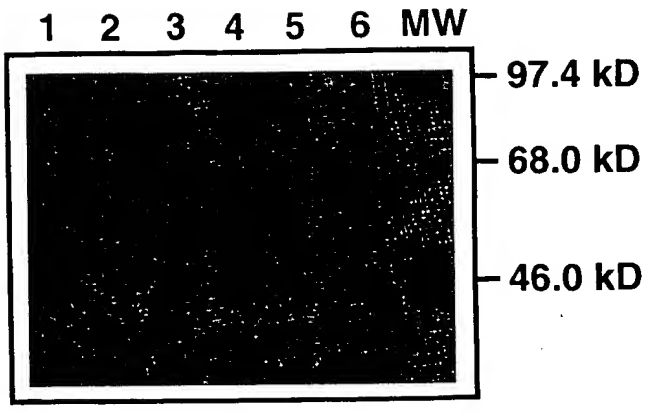

Figure 4. Specific detection of expressed α-gal A protein by Western blot using anti-human α-gal A polyclonal antibody. The specific α-gal A protein migrates at approximately 46 kD. A nonspecific band that also serves as a loading control migrates at <30 kD. Lanes: 1 = mock-transduced normal adherent cells from culture 1; 2 = mock-transduced Fabry adherent cells from culture 1; 3 = transduced Fabry adherent cells from culture 1; 4 = mock-transduced normal adherent cells from culture 2; 5 = mock-transduced Fabry adherent cells from culture 2; 6 = transduced Fabry adherent cells from culture 2; MW = molecular weight marker.

mock-transduced Fabry patient cells. This indicates that expression of the α-gal A protein is stable over time in these cells.

Functional correction

To evaluate if recombinant retroviral transduction and metabolic cooperativity effects can lead to actual functional correction in test cells, we performed immunofluorescence staining of CTH levels using Fabry fibroblasts [15] and adherent Fabry (Fabry #7) cells from the LTBMC. For the staining of transduced Fabry fibroblasts, untouched Fabry fibroblasts and fibroblasts from a normal volunteer were used as controls. We also tested the effects of uptake of secreted α-gal A protein into untouched Fabry fibroblasts. Unconcentrated and unpurified cell supernatant, obtained after 72 hours of incubation with transduced Fabry fibroblasts and filtered, was used as an enzyme source. For this uptake study, naive Fabry fibroblasts were cultivated with this media, containing the secreted α-gal A protein, for 36 hours and then stained for CTH. For the staining of transduced Fabry (Fabry #7) adherent cells, mock-transduced Fabry (Fabry #7) adherent cells and mock-transduced normal (Normal #5) adherent cells were used as controls.

Figure 5 shows the immunofluorescence staining of CTH in fibroblasts. Normal fibroblasts show little background staining (Fig. 5A). In Fabry fibroblasts, accumulated CTH was clearly detected in a broad distribution pattern throughout cells (Fig. 5B), but in transduced Fabry fibroblasts, clearance of accumulated CTH was observed (Fig. 5C). Clearance of accumulated CTH also was confirmed in Fabry fibroblasts that took up the enzyme from the media (Fig. 5D), demonstrating that metabolic cooperativity leads to a functional effect.

Figure 6 shows the immunofluorescence staining of CTH in adherent cells from LTBMC at 10 weeks. Normal adherent cells that were mock-transduced are shown in Figure 6A. In Fabry adherent cells, accumulated CTH was clearly detected (Fig. 6B), but in transduced Fabry adherent cells, clearance of accumulated CTH was again apparent (Fig. 6C).

Discussion

The present preclinical investigations demonstrate that transduction of enriched populations of primitive hematopoietic and stromal cells derived from patients with Fabry disease is feasible with a therapeutic recombinant retrovirus that contains the coding sequence for human α-gal A. This transduction leads to intracellular enzymatic correction in bone marrow-derived cells, an important population of cells that has impact for the clinical management of this disorder. Transduction also leads to extracellular secretion from these target cells. In addition, we demonstrate that transduction and enzymatic uptake lead to functional correction of some target cells as measured by a marked decrease in intracellular CTH levels. To our knowledge, this is the first report of specific long-term functional correction with gene transfer for this disorder. Taken together, these results point to the possibility that transduced hematopoietic bone marrow cells can become long-lived and stable systemic enzyme delivery vehicles that are able to deliver the corrective factor to a number of tissues. This procedure might be especially beneficial if these cells were combined in clinical protocols with cells such as lymphocytes that can be transduced [15] and expanded prior to implantation. Even if the overall amount of circulating enzyme produced systemically is low, it is possible that these delivery vehicles may provide beneficial quantities of enzyme at clinically important sites.

Using the hematopoietic system for systemic delivery may have other advantages over various other treatment strategies proposed for this disorder. In contrast to intravenous infusion of enzyme, single site-restricted implantation of transduced and secreting cells, or direct injection of DNA into muscle cells [27], circulating monocytes, for example, may be able to deliver therapeutic quantities of α-gal A to a greater range of locales. This postulate is based on the fact that such cells can access important areas including the brain, lymph nodes, and lung. Retrovirally transduced cells have been shown to migrate to the brain in vivo [28,29]. This migration and secretion scenario is in contrast with the somewhat limited distribution of enzyme that has been documented in enzyme replacement therapy for Gaucher disease [30]. $CD34^+$ cells have been shown recently to contain progenitors to vascular endothelial cells [12,13]. If these cells can circulate and implant at multiple locales and still demonstrate enzyme secretion, therapy then might directly address the vascular lesions due to lipid accumulation that are prevalent in this disorder.

The results from the transduction of bone marrow cells in

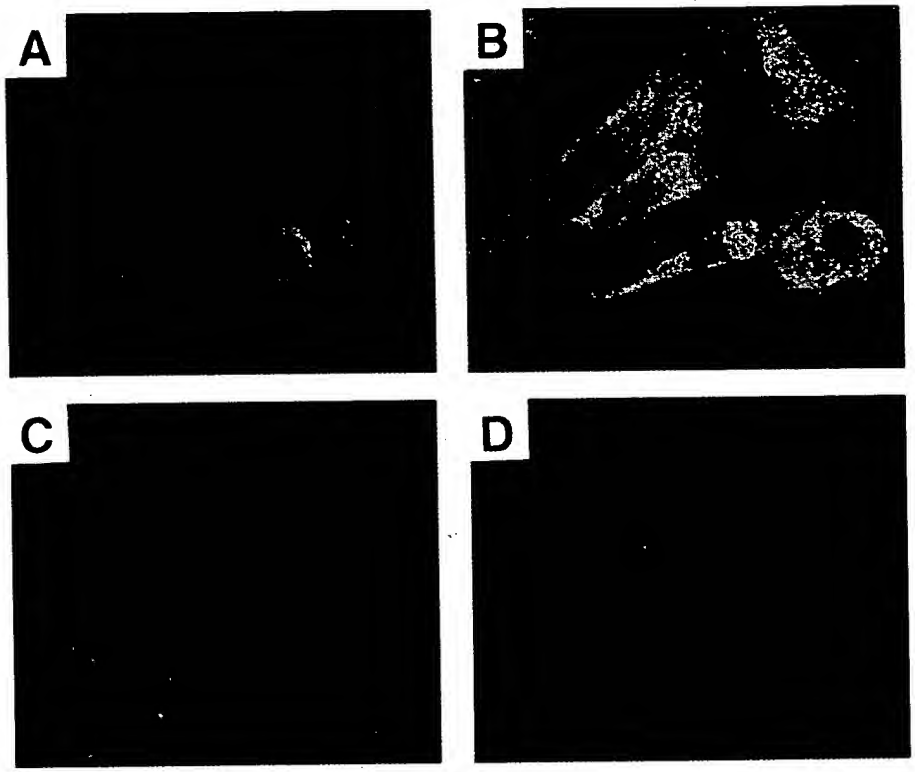

Figure 5. Immunofluorescence staining of human skin fibroblasts with the anti-CTH monoclonal antibody. **(A)** Normal fibroblasts. **(B)** Untouched Fabry fibroblasts. **(C)** Transduced Fabry fibroblasts. **(D)** Fabry fibroblasts after endocytosis of secreted α-gal A.

the LTBMCs are encouraging, as they show that intracellular correction can be maintained as cells age and differentiate and that corrected cells can secrete enzyme activity for an extended period of time. The α-gal A enzyme in media appears to be stable over time or, alternatively, the secretion/turnover levels are high enough to generate sustained secretion levels in the media. Slight differences were seen when different transduction schedules were used on the cells in the LTBMC. Transduction schedule 1 [21], with one less transduction by recombinant virus, gave somewhat higher enzyme activities over time and used less manipulations than transduction schedule 2 [22], which could damage cells and also compromise culture sterility. To extend these studies, much longer culture times will be necessary to fully evaluate whether the most primitive repopulating stem cells have been transduced or another more exhaustive assay system, such as SCID mouse models for human hematopoiesis [31], needs to be explored.

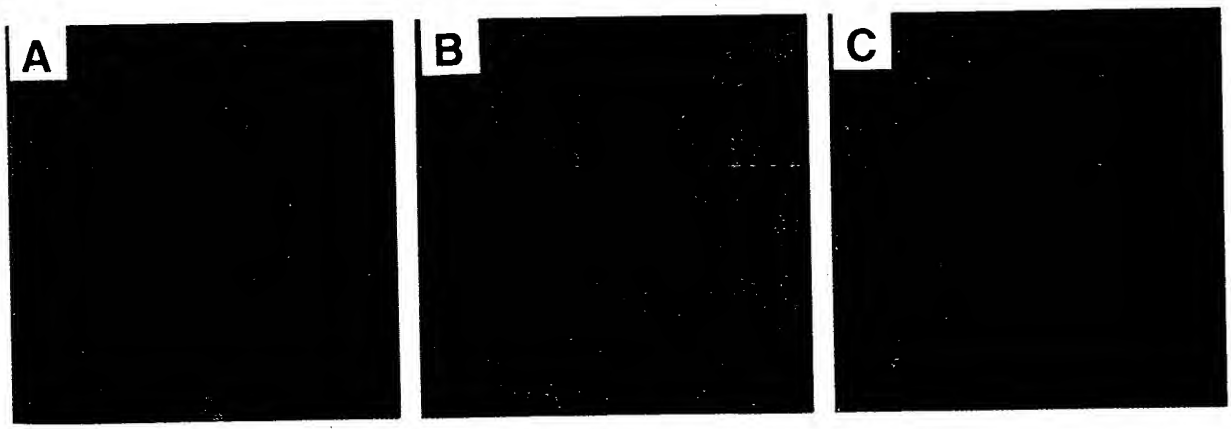

Figure 6. Immunofluorescence staining, with the anti-CTH monoclonal antibody, of human 10-week long-term bone marrow culture (LTBMC) adherent cells. **(A)** Mock-transduced normal LTBMC adherent cells. **(B)** Mock-transduced Fabry LTBMC adherent cells. **(C)** Transduced Fabry LTBMC adherent cells.

In actuality, the transduction and long-term culture/expansion protocol may have direct clinical effects and may be an effective approach for therapy in itself. This strategy has been proposed as an alternative method for effecting corrective gene transfer [21,32,33] to the ex vivo transduction of $CD34^+$-enriched cells that has been used in some initial clinical trials. Human bone marrow aspirates contain a variety of cells, including fibroblast-like cells and stromal cells that contain precursors to bone, adipocytes, chondrocytes, and myoblasts that can likely be readily transduced by retroviral vectors [34]. These transduced cells may then be expanded and reinfused into the patient [35], although there remains some controversy about the effectiveness of this transplantation [36,37]. In this way, this correction may provide initially high levels of systemic enzyme activity due to transfected marrow stromal cells while differentiation and repopulation of the hematopoietic system by the transduced stem/progenitor cells occurs. Transduction of LTBMC has been done in the context of Hurler syndrome, and long-term enzymatic correction and secretion into the media were observed [22].

Important preclinical studies on gene transfer for Fabry disease in animal models are now possible, because an α-gal A deficient mouse recently has been generated [38]. Furthermore, if transduction efficiencies or enzyme levels remain too low for beneficial effects, it may be possible to increase the levels of expression by altering the viral or α-gal A constructs or by sorting for transduced cells prior to implantation. Viral constructs incorporating cell surface selectable markers [16] along with the therapeutic α-gal A gene have been initiated to facilitate these studies.

Acknowledgments

The authors are grateful to Dr. Cecilia Fahlman (University of Lund, Sweden) for advice on the LTBMCs, Dr. Carolyn Smith (NINDS, NIH) for help with the fluorescence microscopy, and to Drs. Harry Malech (NIAID, NIH) and Stefan Karlsson (University of Lund, Sweden) for discussions.

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AU Takenaka, Toshihiro; Hendrickson, Chad S.; Tworek, David M.; Tudor, Matthew; Schiffmann, Raphael; Brady, Roscoe O.; Medin, Jeffrey A.
TI Enzymatic and functional correction along with long-term enzyme secretion from transduced bone marrow hematopoietic stem/progenitor and stromal cells derived from patients with Fabry disease
SO Experimental Hematology (New York) (1999), 27(7), 1149-1159.

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L6 ANSWER 75 OF 94 MEDLINE DUPLICATE 31
AU Ohsugi K; Kobayashi K; Itoh K; Sakuraba H; Sakuragawa N
TI Enzymatic corrections for cells derived from Fabry disease patients by a recombinant adenovirus vector.
SO JOURNAL OF HUMAN GENETICS, (2000) 45 (1) 1-5.
Journal code: 9808008. ISSN: 1434-5161.

L6 ANSWER 76 OF 94 MEDLINE DUPLICATE 32
AU Takiyama N; Dunigan J T; Vallor M J; Kase R; Sakuraba H; Barranger J A
TI Retrovirus-mediated transfer of human alpha-galactosidase A gene to human CD34+ hematopoietic progenitor cells.
SO HUMAN GENE THERAPY, (1999 Dec 10) 10 (18) 2881-9.
Journal code: 9008950. ISSN: 1043-0342.

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J Hum Genet (2000) 45:1–5

ORIGINAL ARTICLE

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Enzymatic corrections for cells derived from Fabry disease patients by a recombinant adenovirus vector

Received: July 27, 1999 / Accepted: September 16, 1999

Abstract Fabry disease is an X-linked inherited metabolic disorder caused by a deficiency of α-galactosidase (α-gal), resulting in the accumulation of ceramide trihexoside (CTH) in body fluids and in many organs and tissues. We constructed a recombinant adenovirus with a human α-gal cDNA (AxCAG α-gal), and transfected this vector to skin fibroblasts from Fabry patients. Transfected cells expressed high amounts of α-gal in their cytoplasm, and a high level of α-gal activity was detected in the medium. The accumulated CTH in the fibroblasts disappeared 3 days after infection. The secreted α-gal also eliminated the accumulated CTH from uninfected patient's cells. The enzyme may be taken up through mannose-6-phosphate receptors, as the addition of mannose-6-phosphate to the medium completely inhibited the uptake of the enzyme. The infected cells continued to express α-gal for more than 10 days. These results suggest that AxCAG α-gal could be used as enzyme replacement gene therapy for Fabry disease.

Key words α-Galactosidase · Fabry disease · Adenovirus vector · Trihexoside · Gene therapy

Introduction

Fabry disease is an X-linked inherited glycolipid metabolic disorder resulting from deficient activity of the lysosomal enzyme, α-galactosidase (EC.3.2.1.22, α-gal). Neutral glycosphingolipids with terminal α-gal-linked galactosyl moieties — globotriaosylceramide (ceramide trihexoside [CTH]) — accumulate in the liver, heart, spleen, kidney, and vascular endothelial cells, as well as in the plasma of patients with Fabry disease. Clinical manifestations of the classical phenotype include angiokeratoma, acroparesthesia, and vascular abnormalities of heart and kidneys, from childhood. A mild phenotype with relatively high residual enzyme activity has been reported as an atypical variant (Sakuraba et al. 1990; Nagao et al. 1991; von Scheidt et al. 1991; Ishii et al. 1992). Treatment strategies for Fabry disease are based on the discovery that lysosomal enzymes are exported from normal cells and are taken up by α-gal deficient cells (Mapes et al. 1970). To date, infusions of the purified enzyme (Brady et al. 1973; Tsuji et al. 1994) and plasma exchange (Pyeritz et al. 1980) have been used as therapies for this disease, but, unfortunately, stable clinical effects have not been achieved. Other types of treatment have been attempted, including kidney transplantation (Clarke et al. 1972) and fetal liver transplantation (Touraine et al. 1979). Long-term observation is necessary for evaluating these treatments. In recent years, gene therapy has been suggested as a curative therapy for lysosomal diseases. In most gene therapies for Fabry disease, retrovirus vectors have been employed in preclinical studies (Sugimoto et al. 1995; Medin et al. 1996; Ohshima et al. 1997). However, a clinical study has not yet been carried out.

This study describes the construction and employment of a recombinant adenovirus vector to ameliorate the enzymatic defect in skin fibroblasts derived from patients with Fabry disease. Cells engineered to overexpress α-gal were also found to secrete large amounts of α-gal. We found that this enzyme was taken up by the uncorrected cells of patients through mannose-6-phosphate (Man-6-P) receptors.

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Materials and methods

Vector construction

The adenovirus vector, AxCAG α-galactosidase (AxCAG α-gal) was constructed by the Miyake method (Miyake et al.

1996). Adenoviruses lacking the E1 region of their genome are replication-incompetent and are grown in the complementary human embryonic kidney 293 cell line. 293 cells are primary human embryonic kidney cells, transformed by sheared human adenovirus type 5 DNA. A pAx1CAwt cosmid was created from the human adenovirus type 5 (Ad5) genome from which the E1a, E1b, and E3 regions were deleted and replaced with an expression unit containing the CAG promoter, composed of the cytomegalovirus enhancer plus the chicken β-actin promoter, a Swa I site, and the rabbit β-globin polyadenylation signal. Human α-gal cDNA was prepared from cultured lymphoblasts, synthesized and amplified by reverse transcription polymerase chain reaction (RT-PCR) in accordance with the Okumiya method (Okumiya et al. 1995).

Cell culture

Skin fibroblasts were obtained from patients with Fabry disease. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and cultured in a 35-mm petri dish in a humidified 5% CO_2 atmosphere at 37°C.

Fibroblasts from normal volunteers (normal controls) and Fabry patients were infected with AxCAG α-gal at varying multiplicity of infection (MOI). Mock-infected cells were infected with adenovirus vector without transfer gene at an MOI of 50. α-Gal activity, as well as β-hexosaminidase activity, was measured in cells cultured for 3 days after infection.

Enzyme assay

After centrifugation at 5,000 rpm for 5 min, the clear medium was stored at −20°C until assay. The cells were harvested by scraping with a rubber policeman, washed with phosphate-buffered saline (PBS), centrifuged, and stored as pellets at −20°C until assay. The frozen pellets were thawed and agitated with distilled water, using a Vortex mixer (Scientific Industries, Bohemia, NY, USA). The mixture was sonicated three times for 20 s each time, on ice and frozen and thawed three times. These suspensions were assayed as cell extracts.

α-Gal activity was determined using 4-methylumbelliferyl-α-D-galactopyranoside as the substrate in the presence of N-acetyl-galactosamine, an inhibitor of N-acetylgalactosaminidase, by the Mayes method (Mayes et al. 1981). In addition, β-hexosaminidase activity (Tallman et al. 1974) was measured, using cell extracts and media as an enzyme control. Protein concentration was determined by Lowry's method (Lowry et al. 1951).

CTH immunofluorescence staining

According to the Itoh method (Itoh et al. 1993), fibroblasts (1×10^4) were cultured on cover slips (LAB-Tek chamber slide; Nunc, Naperville, IL, USA), infected with AxCAG α-gal. After being cultured, cells were fixed with 2% paraformaldehyde on ice, then washed three times with PBS, blocked for nonspecific binding with bovine serum albumin, and treated with monoclonal mouse IgG anti-CTH (Kotani et al. 1992) as a first antibody and with fluorescein isothiocyanate (FITC)-conjugated F(ab)$_2$ gout anti-mouse IgG as a second antibody. The stained cells were examined with a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad, Hemel Hempstead, UK) attached to a Nikon microscope (Optiphot 2; Nikon, Tokyo, Japan).

Uptake and secretion studies

In fibroblasts, lysosomal enzymes may be transported from the extracellular space to lysosomes through receptor-mediated endocytosis. The uptake of the secreted enzymes was almost completely blocked when Man-6-P was added to the incubation medium indicating that the uptake was mediated through the Man-6-P receptors on these cells. One-mM Man-6-P has usually been employed for uptake studies of the secreted enzyme (Medin et al. 1996). Accordingly, the cells were cultured in medium containing 1 mM Man-6-P, followed by immunofluorescence staining to detect accumulated CTH in the cells.

Results

Adenovirus construct

The recombinant adenovirus Ax CAG α-gal construct is shown in Fig. 1. We tested the ability of AxCALacZ (recombinant adenovirus with inserted *Escherichia coli LacZ* gene) to infect the fibroblasts. The cells were infected with AxCALacZ at an MOI of 5, 20, and 50. About 6.6%, 33%, and 80% of the cells were positive for β-gal activity at an MOI of 5, 20 and 50, respectively.

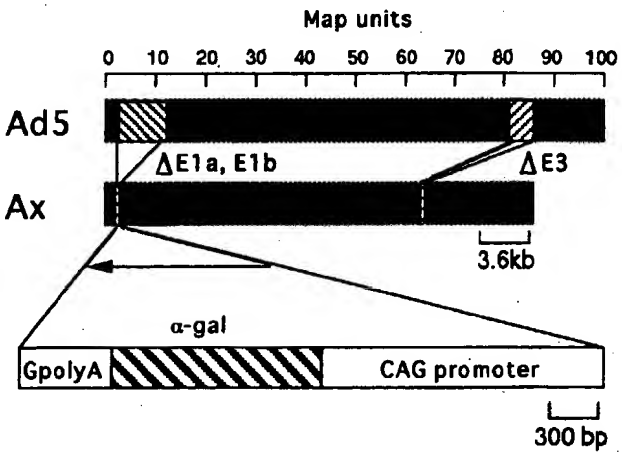

Fig. 1. Construct of the recombinant adenovirus vector (AxCAG α-gal). AxCAG α-gal was constructed by inserting the human α-gal cDNA and CAG promoter into human adenovirus type 5 genomes with deleted E1a, E1b, and E3 regions

Enzymatic correction of cells from patients with Fabry disease

α-Gal activity in Fabry fibroblasts was less than 10% that of normal controls; 14.7 ± 5.9 (normal controls) and 0.5 ± 0.3 nmol/mg per h (patients). The β-hexosaminidase activity of the cells was within normal limits; 470.3 ± 162.5 (normal controls), and 461.3 ± 119.4 nmol/mg per h (patients). The cells infected with AxCAG α-gal showed greatly increased α-gal activity depending on MOI (Fig. 2). α-Gal transfected cells (MOI 5) had α-gal activity ten times higher than the normal control levels. No increase in α-gal activity was observed in the mock-infected cells (Fig. 3). β-Hexosaminidase activity was not affected under these conditions.

Immunostaining with CTH antibody is shown in Fig. 4. In the cells from Fabry patients, strong fluorescence was detected, while fluorescence was scarcely detected in normal subjects (Fig. 4A,B). The accumulated CTH in Fabry fibroblasts decreased after infection with AxCAG α-gal and was inversly proportional to MOI (Fig. 4C,D).

Secretion studies

To determine whether extracellular secretion of α-gal occurred in the corrected cells, the cells from Fabry patients were infected with AxCAG α-gal at an MOI of 50 and were cultured in DMEM with 5% FCS to suppress cell growth. The α-gal activity in cells and medium was measured for 10 days. The enzyme activity was assayed on day 3 after the medium was changed. α-Gal activity in the cells increased for 7 days and remained at a high level. The enzyme activity in the medium also increased, similarly to findings in the cells (Fig. 5).

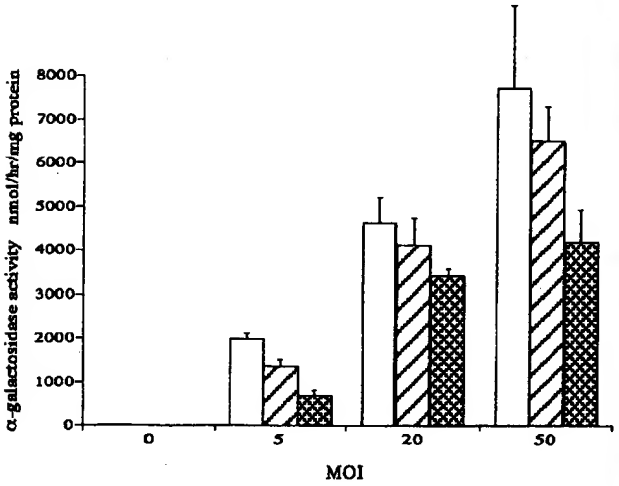

Fig. 2. α-Galactosidase (gal) activity in fibroblasts infected with AxCAG α-gal. Fibroblasts from normal volunteers (control) and patients with Fabry disease were infected with AxCAG α-gal at various multiplicity of infection (*MOI*). α-Gal activity was determined on day 3 after infection. α-Gal activity in uninfected cells was 15.5 ± 3.8 (control; *white bars*), 1.2 ± 0.4 (patient 1; *diagorally cross-hatched bars*), and 0.7 ± 0.2 nmol/h per mg (patient 2, *criss-crossed bars*)

Uptake studies

The uptake of the secreted enzyme by non-treated cells from Fabry patients was examined by immunostaining. Cells were cultured in normal medium for 6 days, and showed extensive accumulation of CTH (Fig. 6A). The cell culture with medium containing secreted α-gal showed markedly decreased CTH accumulation in Fabry cells (Fig. 6B; 25%, Fig. 6C; 50% of medium containing secreted α-gal). The addition of 1 mM Man-6-P to the medium containing secreted α-gal suppressed the removal of CTH

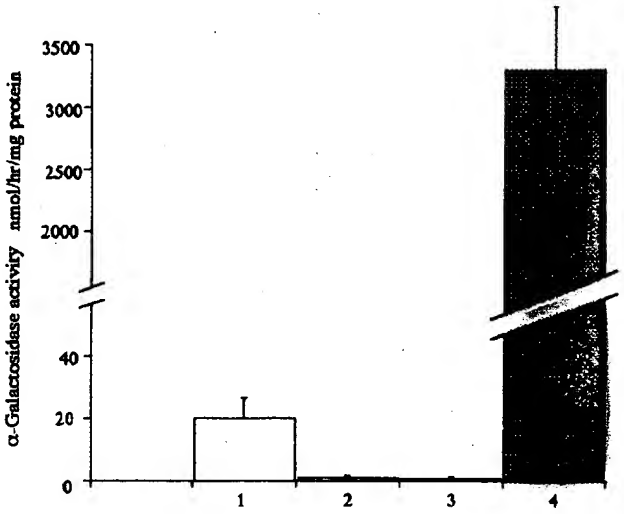

Fig. 3. Correction of enzymatic defect in fibroblasts from Fabry patients. Mock-infected cells were infected at an MOI of 20 and corrected cells were infected at an MOI of 5. *1, White bar*, Control fibroblasts; *2*, patient fibroblasts; *3, patient fibroblasts, mock-infected*; *4, black bar*, patient fibroblasts AxCAG α-gal-infected

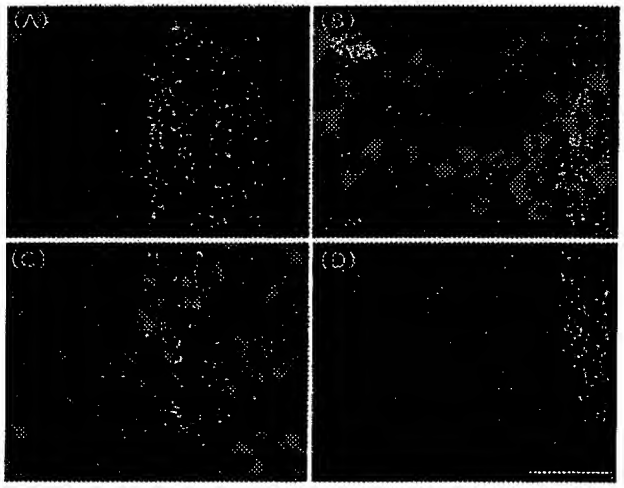

Fig. 4A–D. Immunostaining for ceramide trihexoside (CTH) in Fabry fibroblasts infected with AxCAG α-gal. Fixation and immunostaining were performed after day 3 of infection. **A** Non-treated cells from a control subject. **B** Non-treated cells from a patient. **C** Infected cells from a patient at an MOI of 20. **D** Infected cells from a patient at an MOI of 50. *Scale bar*, 100 μm

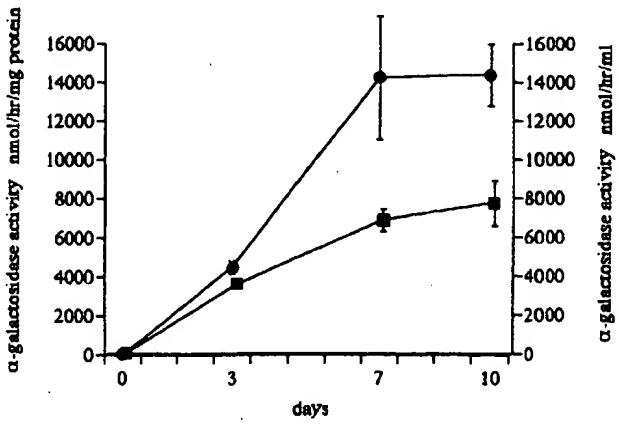

Fig. 5. α-Gal activity of medium with time (*squares*) and the enzyme activity in cells (*closed circles*) from a Fabry patient. The infected cells from the patient were cultured in medium supplemented with 5% fetal calf serum. The medium was changed every 3 or 4 days. Enzyme activity was assayed on day 3 after medium was changed. *Left bar* indicates enzyme activity in cells (nmol/mg per h) and *right bar* shows enzyme activity in the medium (nmol/ml per h)

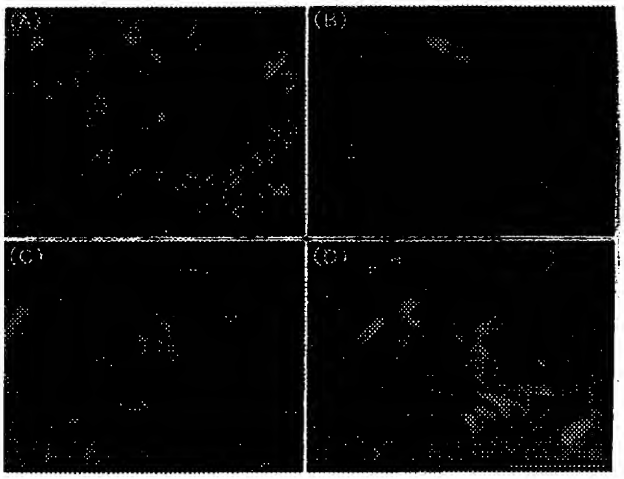

Fig. 6A–D. Immunostaining for CTH in Fabry patient's fibroblasts which had taken up the secreted enzyme. The cells were cultured for 6 days in medium containing a high amount of the secreted enzyme. **A** Cells cultured in normal medium. **B** Cells cultured in medium with 25% by volume of medium containing the secreted enzyme. **C** Cell cultured in medium with 50% by volume of medium containing the secreted enzyme. **D** Cells cultured in the same medium as that in **(C)** but including 1 mM mannose-6-phosphate. *Scale bar*, 200 μm

accumulation in the Fabry cells (Fig. 6D). This result suggested that the secreted enzymes were taken up through the Man-6-P receptors.

Discussion

In this report, we describe the construction of a recombinant adenovirus vector that engineered efficient transduction and the expression of α-gal activity in Fabry cells. This vector eliminated the enzymatic defect in cells obtained from patients with Fabry disease. In addition to the amelioration of the enzyme defect in fibroblasts from the patients, we demonstrated accumulated CTH removal from the uncorrected cells by immunostaining. Further, the transfected cells secreted a high activity of α-gal into the medium, which was taken up by the uncorrected cells in a Man-6-P receptor-specific manner, resulting in the correction of accumulated CTH.

Treatment of Fabry disease has been based on α-gal supplement action. Initially, enzyme therapy was attempted by infusing normal human plasma (Mapes et al. 1970), but results were limited because of the low level of enzyme activity present, the rapid clearance of the infused enzyme, and the hazards of repeated plasma transfusions. Intravenous infusion of purified human α-gal briefly reduced plasma CTH levels (Brady et al. 1973; Desnick et al. 1979). Plasma exchange has also been attempted (Pyeritz et al. 1980). But these treatments have not achieved stable clinical effects. Other attempts were made, including transplantation of kidney (Clarke et al. 1972) and fetal liver (Touraine et al. 1979). Long-term observation is necessary for deciding the feasibility of these methods. Tsuji et al. (1994) was able to deliver the purified enzymes to target cells by using α_2-macroglobulin as the transport vehicle.

In recent years, gene therapy has been expected to be a curative therapy for lysosomal disease. Gene therapies for Fabry disease have been studied by using retroviruses. Sugimoto et al. (1995) reported the efficient expression of human α-gal in NIH3T3 cells, using a bicistronic multidrug-resistant gene retrovirus. Medin et al. (1996) described the construction and application of a high-titer recombinant retroviral vector to correct the enzymatic defect in hematopoietic and other cells derived from patients with Fabry disease. Their results were effective for correcting the enzyme deficiency in the cells from these patients in vitro.

A recombinant adenovirus vector has very high titers and can transduce the gene into both divided and undivided cells. Our AxCAG α-gal expressed more than 100-fold the α-gal activity of an AxCAG α-gal infection at an MOI of 5, compared with uninfected cells, although the infection rate was only 6% at an MOI of 5. The expression of α-gal activity by AxCAG α-gal started 24 h after infection, increased gradually for 7 days, and remained at a high level for more than 10 days. The accumulated CTH in the cells from the patients disappeared 3 days after AxCAG α-gal infection at an MOI of 50. α-Gal activity was very high in the cells and was secreted extensively into the medium, where this enzyme was taken up by uncorrected cells. It is not clear whether very high α-gal activity exerts an influence on cells or tissues. Kase et al. (1998) reported that transgenic mice expressing human α-gal had α-gal activity of 22, being approximately 11,080-fold higher in the liver, kidney, heart, and other tissues of these mice than that in non-transgenic mice. Kase et al. did not describe the clinical disadvantages of the transgenic mice. Ioannou et al. (1996) used enzyme replacement therapy in α-gal-deficient mice, with resultant reduction in the substrate levels in plasma, liver, and heart. Ohshima et al. (1997) established α-gal gene knockout mice and showed that they were useful as Fabry disease models.

More recently, they reported that aging accentuated and bone marrow transplantation ameliorated metabolic defects in their knockout mice (Ohshima et al. 1999).

Our data provided compelling evidence that the recombinant adenovirus vector corrected the accumulated substrate, CTH, in fibroblasts from Fabry patients. Judging from the evidence showing the uptake of secreted enzyme, which also decreased CTH, we suggest that our therapeutic system, using recombinant adenovirus, could be employed for cell therapy using genetically modified stem/progenitor cells from normal and Fabry patient donors.

Acknowledgments We thank Dr. Satoru Takahashi for his great help and many suggestions, and Ms. Keiko Kawanishi for assaying the enzymes. This work was supported in part by Health Sciences Research Grants for Research on Brain Science and Research on Advanced Medical Technology.

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TI Enzymatic and functional correction along with long-term enzyme secretion from transduced bone marrow hematopoietic stem/progenitor and stromal cells derived from patients with Fabry disease
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AU Takiyama N; Dunigan J T; Vallor M J; Kase R; Sakuraba H; Barranger J A
TI Retrovirus-mediated transfer of human alpha-galactosidase A gene to human CD34+ hematopoietic progenitor cells.
SO HUMAN GENE THERAPY, (1999 Dec 10) 10 (18) 2881-9.
Journal code: 9008950. ISSN: 1043-0342.

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HUMAN GENE THERAPY 10:2881–2889 (December 10, 1999)
Mary Ann Liebert, Inc.

Retrovirus-Mediated Transfer of Human α-Galactosidase A Gene to Human CD34$^+$ Hematopoietic Progenitor Cells

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ABSTRACT

Fabry disease, caused by a deficiency of lysosomal enzyme α-galactosidase A (α-gal A), is one of the inherited disorders potentially treatable by gene transfer to hematopoietic stem cells. In this study, a high-titer amphotropic retroviral producer cell line, MFG-α-gal A, was established. CD34$^+$ cells from normal umbilical cord blood were transduced by centrifugal enhancement. The α-gal A activity in transduced cells increased 3.6-fold above the activity in nontransduced cells. Transduction efficiency measured by PCR for the integrated α-gal A cDNA in CFU-GM colonies was in the range of 42–88% (average, 63%). The expression of functional enzyme in TFI erythroleukemia was sustained for as long as cells remained in culture (84 days) and for 28 days in LTC-IC cultures of CD34$^+$ cells. The ability of the transduced CD34$^+$ cells to secrete the enzyme and to correct enzyme-deficient Fabry fibroblasts was assessed by cocultivation of these cells. The enzyme was secreted into the medium from transduced CD34$^+$ cells and taken up by Fabry fibroblasts through mannose 6-phosphate receptors. These findings suggest that genetically corrected hematopoietic stem/progenitor cells can be an enzymatic source for neighboring enzyme-deficient cells, and can potentially be useful for gene therapy of Fabry disease.

OVERVIEW SUMMARY

To assess the potential for *ex vivo* gene therapy for Fabry disease, cord blood CD34$^+$ cells were transduced with a retroviral vector, MFG-α-galactosidase A. The enzyme secreted from transduced CD34$^+$ cells corrected enzyme-deficient Fabry fibroblasts, suggesting the feasibility of treating patients with Fabry disease by gene transfer into hematopoietic stem/progenitor cells.

INTRODUCTION

FABRY DISEASE is an X-linked recessive lysosomal storage disorder. It is caused by a deficiency of the lysosomal enzyme α-galactosidase A (α-gal A), which catalyzes the degradation of glycosphingolipids with terminal α-galactosyl residues. This defect results in the accumulation of the substrate, especially in vascular endothelium. Clinical manifestations include hypohidrosis, angiokeratoma, acroparesthesia, and corneal opacity. Vascular diseases of the heart, kidneys, and central nervous system develop in adolescence and often lead to an early death (Desnick *et al.*, 1995). There is no specific therapy for this disease although clinical trials of enzyme therapy are in progress.

It is known that the uptake of purified α-gal A enzyme by Fabry fibroblasts leads to correction of the enzyme deficiency *in vitro* (Mayes *et al.*, 1982). This finding suggested the possibility of enzyme replacement therapy for this disease. Several clinical trials of enzyme replacement have been attempted (Brady *et al.*, 1973; Desnick *et al.*, 1979). The infused enzyme activity was rapidly cleared from the circulation. However, large amounts of the purified enzyme were not available for study of this approach. In addition, life-long enzyme therapy will be costly. Other therapeutic approaches including the transplantation of kidneys (Ramos and Tisher, 1994) and fetal livers (Touraine *et al.*, 1979) have not been beneficial.

Gene transfer into hematopoietic stem/progenitor cells may be an alternative approach for the treatment of Fabry disease. Even though the enzyme deficiency in hematopoietic cells is

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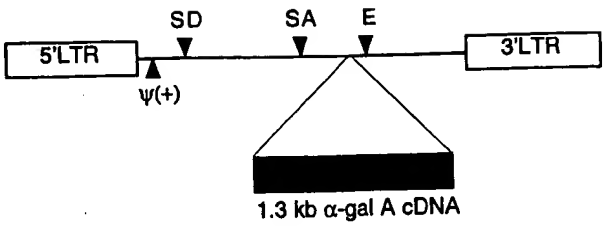

FIG. 1. The MFG-α-gal A plasmid. The α-gal A cDNA is positioned at the start codon of the deleted retroviral envelope gene. Transcription is driven by the endogenous promoter in the 5′ LTR. E, *Eco*RI; SD, splice donor; SA, splice acceptor; Ψ(+), packaging sequence.

not a primary problem, genetically modified hematopoietic stem/progenitor cells can be a continuous, systemic source of active enzyme. The same approach has been tested for the treatment of some mucopolysaccharidoses such as Sly syndrome and Hurler syndrome (Wolfe *et al.*, 1992; Fairbairn *et al.*, 1996). In this study, a high-titer retroviral vector containing the α-gal A CDNA was constructed and used to transduce human $CD34^+$ hematopoietic progenitor cells, using centrifugal enhancement (Bahnson *et al.*, 1995). The methods provide a high level of transduction efficiency. We examined the ability of the transduced $CD34^+$ cells to express and release α-gal A enzyme as well as its uptake by Fabry fibroblasts. The results suggest the potential usefulness of gene transfer to hematopoietic cells as a treatment of Fabry disease.

MATERIALS AND METHODS

Retroviral vector and producer cells

The MFG vector has been previously described (Ohashi *et al.*, 1992). The human α-gal A cDNA-containing plasmid pCXN2Gal (Ishii *et al.*, 1993) was digested with *Eco*RI to produce a 1.3-kb fragment. The ends were filled in by Klenow polymerase and *Bcl*I linkers were added by blunt-end ligation. After *Bcl*I–*Bss*HH digestion, the α-gal A cDNA was ligated to the *Bam*HI–*Nco*I-digested MFG retroviral plasmid, using a 36-bp oligonucleotide (aGAL1, 5′-CAT GCA GCT GAG GAA CCC AGA ACT ACA TCT GGG CTG; aGAL2, 5′-CGC GCA GCC CAG ATG TAG TTC TGG GTT CCT CAG CTG). This strategy placed the cDNA at the start codon of the deleted retrovirus envelope gene to produce MFG-α-gal A (Fig. 1).

The plasmid form of the vector, MFG-α-gal A, was transiently transfected into BOSC 23 cells (Pear *et al.*, 1993), from which were obtained ecotropic vector-containing supernatants 48 hr after transfection. These supernatants were used to cross-infect an amphotropic packaging cell line, Ψ-crip (Danos and Mulligan, 1988). Clones were obtained from the infected polyclonal producer cells by limiting dilution without selection. The culture media from these clones were screened by infection of 3T3 targets. A clone, MT 18, which yielded the highest enzyme expression in target cells, was tested for replication-competent retrovirus (RCR). Both BAG assay and polymerase chain reaction (PCR) for amphotropic envelope were negative (Ohashi *et al.*, 1992; Cornetta *et al.*, 1993). Clone MT 18 was expanded for use in the following experiments.

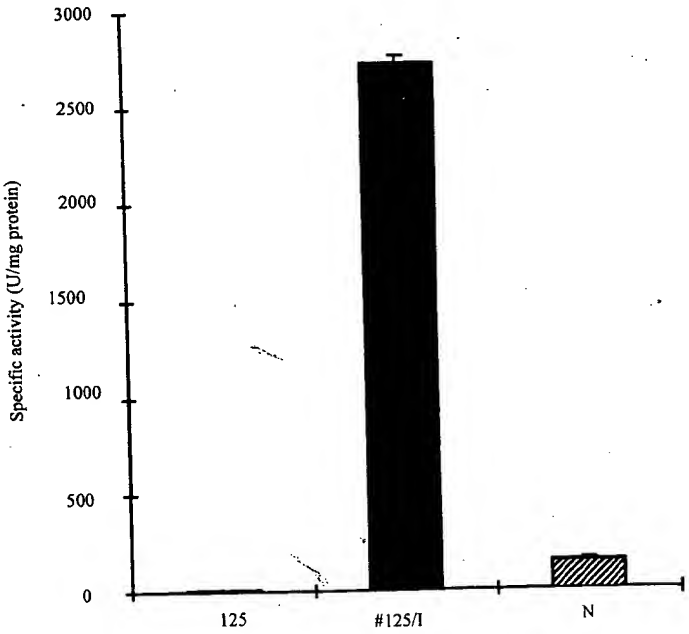

FIG. 2. Expression of α-gal A enzyme in transduced Fabry fibroblasts. Fibroblasts were transduced with MFG-α-gal A, using a 2-hr centrifugation, and assayed for enzyme activity 48 hr after transduction. #125, Fibroblasts from a patient with classic Fabry disease; #125/I, Fabry fibroblasts transduced with MFG-α-gal A; N, fibroblasts from a normal individual.

Cell culture

Primary skin fibroblasts from a patient with classic Fabry disease (patient 125) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Bethesda, MD) containing 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD), 2 m*M* L-glutamine and penicillin–streptomycin (100 U/ml). TFI cells, a factor-dependent human erythroleukemia cell line (American Type Culture Collection [ATCC], Rockville, MD), were cultured in RPMI 1640 (GIBCO-BRL) containing 10% fetal bovine serum, 2 m*M* L-glutamine, penicillin–streptomycin (100 U/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF, 5 ng/ml; Immunex, Seattle, WA). Cord blood (CB) was obtained from discarded placentas after normal deliveries with informed consent and internal review board (IRB) approval. Mononucleated cells were purified from CB using a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient and enriched for CD34$^+$ cells by an immunoaffinity column (Ceprate LC; CellPro, Bothell, WA) as previously described (Mannion-Henderson *et al.*, 1995). CD34$^+$ cells were maintained in long-term bone marrow culture medium (LTBMCM), which consisted of 15% fetal bovine serum, 15% horse serum (GIBCO-BRL), 1 μM hydrocortisone succinate (Abbott Laboratories, North Chicago, IL) 50 μM monothioglycerol, and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO) in Iscove's modified Dulbecco's medium (IMDM; Life Technologies).

Transduction

Fibroblasts were plated at 2.5×10^5 cells/well in six-well plates prior to infection. At the time of infection, medium was

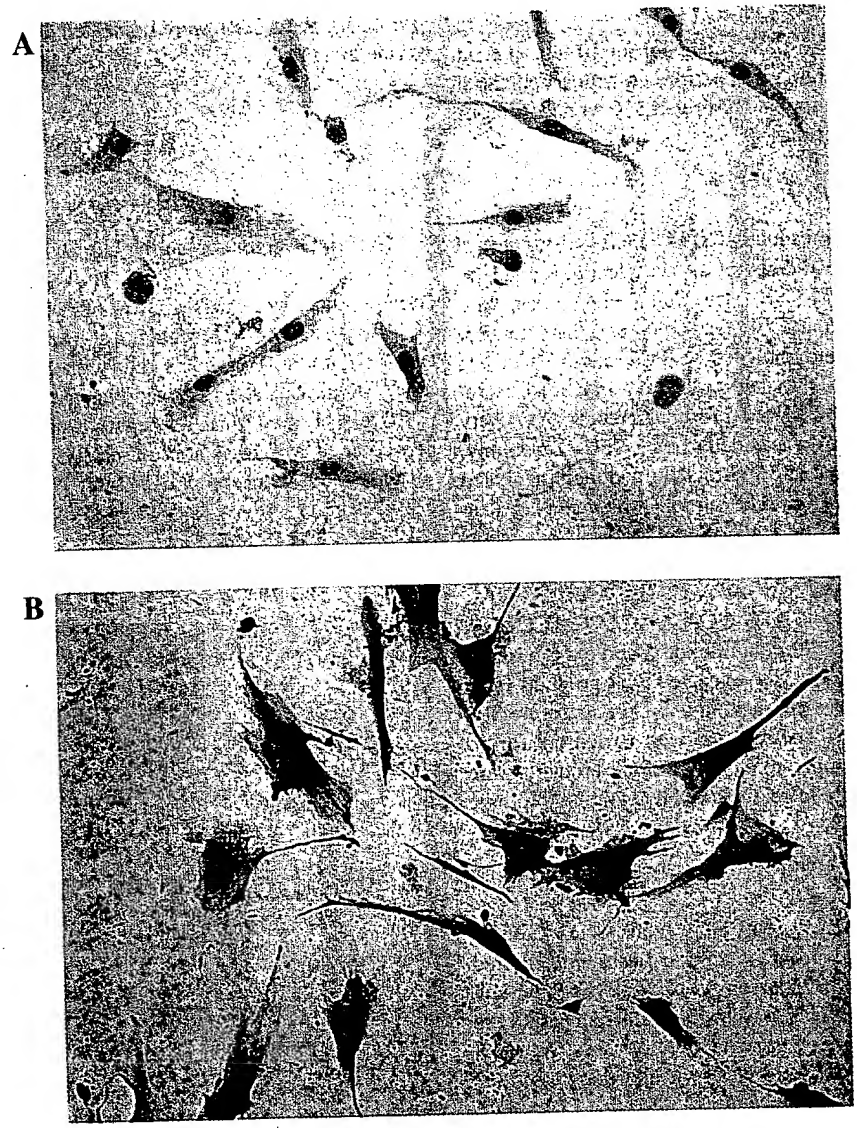

FIG. 3. Immunocytochemical staining of Fabry fibroblasts from patient 125#. Fibroblasts were stained 48 hr after transduction. Cells were incubated with primary antibody (rabbit anti-human-α-gal A antiserum) and then incubated with secondary antibody (goat biotinylated anti-rabbit IgG). This was followed by immunoperoxidase detection. Nuclei were counterstained with hematoxylin solution (**A**) Nontransduced Fabry fibroblasts. (**B**) Fabry fibroblasts transduced with MFG-α-gal A.

replaced with viral supernatant containing Polybrene (8 μg/ml). The cells were transduced by centrifugation at 2400 $\times$ *g* for 2 hr at 24°C (Bahnson *et al.*, 1995). TFI cells and CD34$^+$ cells were transduced in 15-ml culture tubes. CD34$^+$ cells at a concentration of 2 $\times$ 10^5 cells/ml were prestimulated with 10 ng/ml each of interleukin 3 (IL-3), interleukin 6 (IL-6), and stem cell factor (SCF) (Immunex) in LTBMCM for 24 hr. The cells (3 $\times$ 10^5) were transferred into 15-ml tubes to which was added an equal volume of LTBMCM containing MFG-α-gal A retroviral vector and protamine sulfate (Sigma) at 4 μg/ml. The cells were transduced by centrifugation at 2400 $\times$ *g* for 2 hr at 24°C, as described previously (Takiyama *et al.*, 1998).

Immunocytochemistry

Transduced and nontransduced primary fibroblasts, and MT 18 producer cells, were immunostained with polyclonal rabbit antiserum against the purified human α-gal A (Ishii *et al.*, 1994).

The cells were fix in absolute methanol for 10 min at room temperature. Immunostaining was performed with a VectaStain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer instructions.

CFU-GM assay and PCR

Transduced and nontransduced CD34$^+$ cells were plated at a concentration of 1 $\times$ 10^5 cells/ml in methylcellulose supplemented with IL-3 and GM-CSF (Stem Cell Technologies, Vancouver, Canada). Individual CFU-GM colonies generated after 14 days were plucked and analyzed by PCR for the presence of α-gal A transgene. DNA was prepared from individual colonies using Chelex 100 resin (Bio-Rad, Hercules, CA). Fifty microliters of 10% Chelex was added to each CFU-GM colony. The sample was incubated at 56°C for 1 hr and then incubated in boiling water for 10 min. The sample was centrifuged at 12,000 $\times$ *g* for 5 min. Fifteen microliters of the DNA supernatant was used for PCR analysis.

PCR primers were DRT1 (5′-ATA CAG AAA TCC GAC AGT AC-3′) and DRT2 (5′-ATG GCA ATT ACG TCC TTA TC-3′), which hybridize within both exon 5 and exon 6 of the α-gal A genome. The sizes of the expected PCR products from the MFG-α-gal A provirus and genomic α-gal A gene are 309 and 527 bp, respectively. The reaction was carried out for 30 cycles at an annealing temperature of 56°C. PCR products were resolved on a 6% acrylamide gel followed by ethidium bromide staining.

LTC-IC cultures

Transduced and nontransduced CD34$^+$ cells were plated on irradiated, allogeneic BM stroma in 25-cm^2 flasks and cultured for 4 weeks (Mannion-Henderson *et al.*, 1995). Medium (LTBMCM) was changed once a week. Nonadherent cells were removed weekly for α-gal A enzyme activity assay. At week 4, the entire LTC-IC cell culture was trypsinized and used for CFU-GM assay.

Enzyme assay

Cells were sonicated in cold deionized water and assayed for α-gal A activity with 4-methylumbelliferyl-α-D-galactopyranoside (Sigma) as substrate and *N*-acetylgalactosamine (Sigma) as an inhibitor of α-galactosidase B (Mayes *et al.*, 1981). One unit (U) of α-gal A activity hydrolyzed 1 nmol of substrate per hour at 37°C. The amount of protein was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Differences between mean values of the enzyme activity were analyzed by the Student *t* test.

Enzyme uptake studies

Fibroblasts from a Fabry patient (patient 125) were plated at 2.5 $\times$ 10^5 cells per well in a six-well culture plate. Transwells (0.4-μm pore size; Costar, Cambridge, MA) were inserted into the plate. Transduced TFI cells or CD34$^+$ cells were placed into the upper chambers at 2.5 $\times$ 10^5 cells per well, and cocultivated with Fabry fibroblasts placed in the lower chambers to measure enzyme secretion and uptake. To some wells, mannose 6-phosphate was added at a final concentration of 5 m*M*. After 48 hr, fibroblasts were harvested and used for α-gal A enzyme assay.

RESULTS

MFG-α-gal A retroviral vector

The MFG vector containing human α-gal A cDNA was constructed and packaged (Fig. 1). The α-gal A activity of the Ψ-crip producer clone, MT 18, was 60-fold higher than that of the Ψ-crip packaging cells (data not shown). Immunocytochemical analysis of MT 18 producer cells with the anti-human antiserum α-gal A showed strongly positive staining. On the other hand, 3T3 cells showed virtually negative staining (data not shown). The titer of MT 18 is approximately 10^6–10^7 infectious units per milliliter (Bahnson *et al.*, 1994).

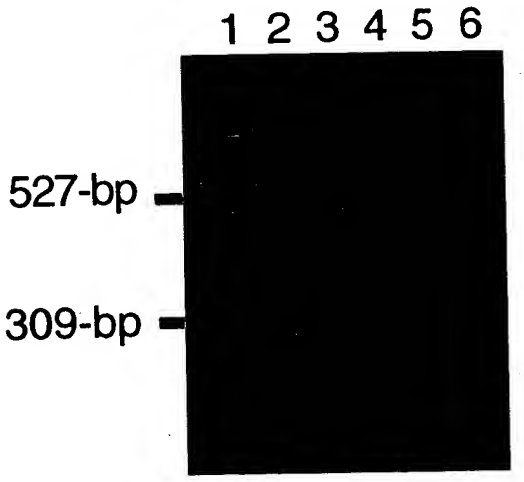

FIG. 4. PCR analysis of CFU-GM colonies. The presence of the α-gal A transgene was detected by PCR with DRT1 and DRT2 primers, which simultaneously amplify both MFG-α-gal A provirus (309 bp) and α-gal A genome (527 bp). Lane 1, size marker (ϕX174/*Hae*III fragments); lane 2, MT 18 producer cells; lane 3, human TFI cells; lane 4, mouse 3T3 cells; lane 5, a transduced colony; lane 6, a nontransduced colony.

TABLE 1. TRANSDUCTION OF CORD BLOOD CD34+ CELLS[a]

CB experiment	α-Gal A activity (U/mg protein)			Transduction efficiency (No. of CFU; positive/total)
	Noninfected	Infected	Fold	
1	320	1732	5.4	88% (21/24)
2	261	904	3.5	58% (14/24)
3	216	430	2.0	42% (10/24)
4	232	766	3.3	63% (15/24)
5	198	736	3.7	67% (16/24)
Average ± SD:	245 ± 48	914 ± 489	3.6 ± 1.2	63% (76/120)

[a]CB CD34+ cells were transduced by 2-hr centrifugation. The cells were assayed for α-gal A enzyme activity 6 days after transduction. They were also assayed by PCR for α-gal A transgene in CFU-GM.

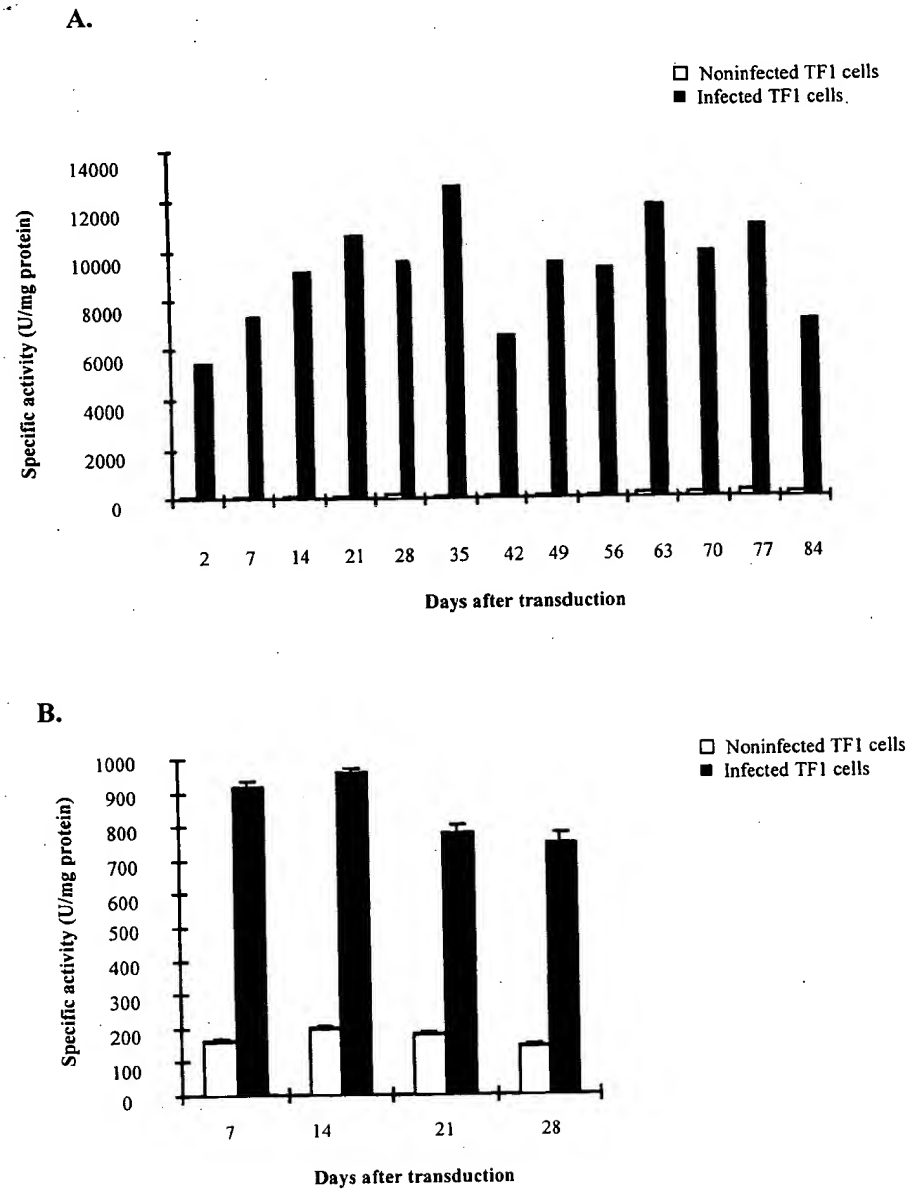

FIG. 5. Long-term expression of α-gal A enzyme in TF1 cells and CB CD34+ cells maintained in LTBMCM after transduction. The histogram compares α-gal A activity in transduced versus nontransduced fractions. (A) Transduced TF1 cells were maintained in culture and assayed for enzyme activity over 84 days. (B) Transduced and nontransduced CB CD34+ cells were maintained in LTBMCM and assayed for enzyme activity over 28 days ($n = 3$).

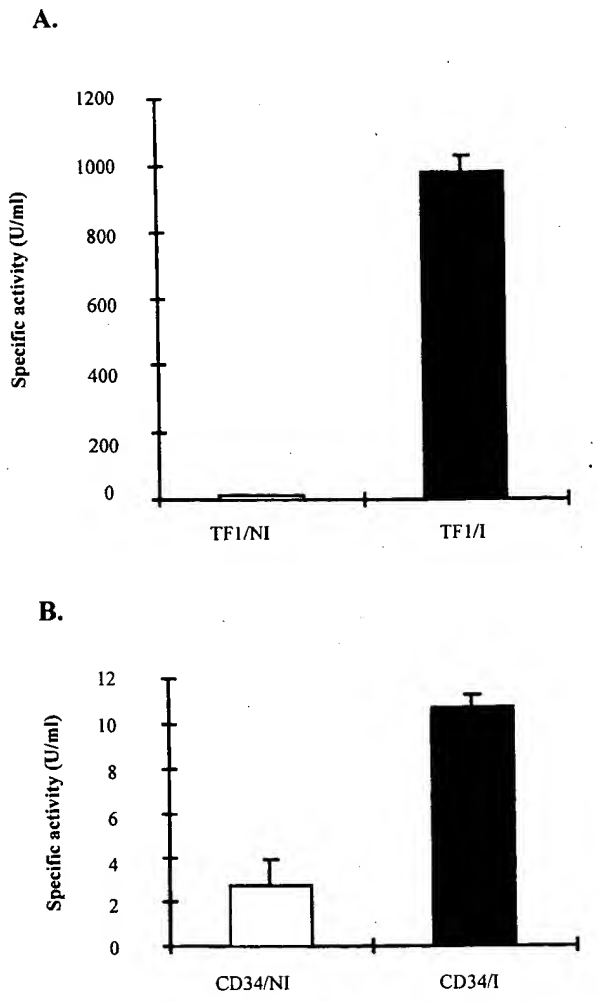

FIG. 6. Secreted α-gal A enzyme in the media of transduced TF1 cells and CD34$^+$ cells. The cells were transduced by 2-hr centrifugation and the media were changed. The supernatants were collected for enzyme assay 48 hr after transduction. (**A**) TF1/NI, the medium of nontransduced TF1 cells; TF1/I, the medium of TF1 cells transduced with MFG-α-gal A. (**B**) CD34/NI, the medium of nontransduced CD34$^+$ cells; CD34/I, the medium of CD34$^+$ cells transduced with MFG-α-gal A.

Transduction of Fabry fibroblasts

Fabry fibroblasts were transduced with MFG-α-gal A, using a 2-hr centrifugation-enhanced infection protocol. The enzyme activity of transduced Fabry fibroblasts increased 340-fold above nontransduced cells, which is about 19-fold above normal fibroblast levels (Fig. 2). Results of immunocytochemical staining with anti-α-gal A antiserum in nontransduced and transduced Fabry fibroblasts are shown in Fig. 3. Transduced cells showed strong staining compared with nontransduced control.

Transduction of CD34$^+$ cells

Cord blood CD34$^+$ cells were transduced by a 2-hr centrifugation-enhanced infection protocol after prestimulation with cytokines (IL-3/IL-6/SCF), and assayed for α-gal A activity. Transduction efficiency was measured by PCR of individual CFU-GM for the presence of the α-gal A transgene (Fig. 4). Results are shown in Table 1. The enzyme activity of CD34$^+$ cells transduced with MFG-α-gal A increased, on average, 3.6-fold above that of nontransduced cells; 914 ± 489 U/mg protein for transduced cells versus 245 ± 48 U/mg protein for nontransduced cells, $p < 0.05$. Transduction efficiency was 42 to 88% (average, 63%).

Long-term expression of α-gal A enzyme

To assess the ability of the α-gal A transgene to sustain expression of a functional enzyme, TF1 cells were transduced and maintained in culture for 84 days. The enzyme activity in transduced TF1 cultures remained at approximately 50-fold above the activity of nontransduced cells (Fig. 5A). Nontransduced and transduced CD34$^+$ cells from normal CB were maintained in LTBMCM for 28 days. The enzyme activity of transduced CD34$^+$ cells remained four- to fivefold above the activity of nontransduced cells (Fig. 5B). CFU-GM colonies resulting from LTCIC (4 weeks) were assayed individually by PCR for the presence of the α-gal A transgene. Four of 16 colonies (25%) were positive for the transgene.

Secretion of α-gal A enzyme from transduced cells and uptake by Fabry fibroblasts

To evaluate the ability of transduced hematopoetic cells to produce and secrete α-gal A enzyme, culture media of transduced TF1 cells and CB CD34$^+$ cells were assayed for enzyme activity. The enzyme activity of media from transduced TF1 cells and CD34$^+$ cells was significantly higher than that from nontransduced cells (3 ± 0.6 versus 981 ± 46 U/ml, $p < 0.01$, for TF1 cells; 2.7 ± 1.2 versus 10.7 ± 0.6 U/ml, $p < 0.01$, for CD34$^+$ cells) (Fig. 6). Transduced TF1 cells and CD34$^+$ cells were cocultivated with Fabry fibroblasts in Transwells for 48 hr, which provided for an estimation of the secreted enzyme to correct the metabolic defect within the cells. The enzyme activity of Fabry fibroblasts cocultivated with transduced TF1 cells and CD34$^+$ cells increased 59- and 3.4-fold, respectively. This uptake was completely blocked in the presence of 5 m*M* mannose 6-phosphate (Fig. 7).

DISCUSSION

This study demonstrated the following: (1) a high-titer recombinant retrovirus carrying the α-gal A gene was produced; (2) human CD34$^+$ hematopoietic progenitor cells were efficiently transduced; (3) expression of the α-gal A transgene was sustained in cell cultures; (4) transduced CD34$^+$ cells expressed and secreted α-gal A enzyme into the medium; and (5) secreted enzyme was taken up by enzyme-deficient Fabry fibroblasts through a mannose 6-phosphate receptor-mediated mechanism.

The potential of gene therapy for Fabry disease has been evaluated (Sugimoto *et al.*, 1995; Medin *et al.*, 1996; Novo *et al.*, 1997). Medin *et al.* reported that the secreted α-gal A enzyme corrected the deficiency in Fabry B cell lines and fibroblasts, and Novo *et al.* reported that the enzyme from mouse myoblasts transfected with α-gal A cDNA corrected the enzyme deficiency in Fabry fibroblasts. In our study, we have

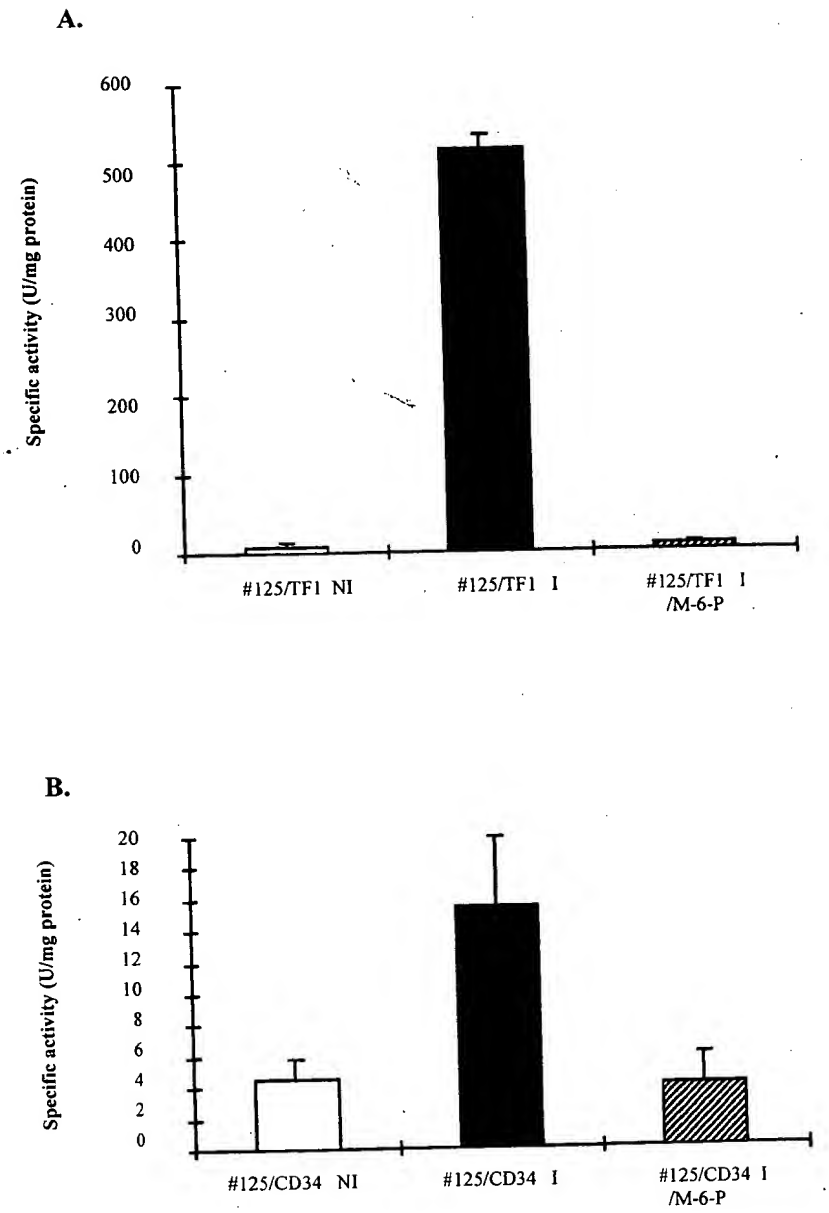

FIG. 7. Uptake by Fabry patient fibroblasts of α-gal A enzyme secreted by transduced TF1 cells or CD34+ cells. The upper chamber of the Transwell contained 2.5×10^5 transduced TF1 cells (**A**) or CD34+ cells (**B**), which can secrete α-gal A enzyme, and the lower chamber contained 2.5×10^5 Fabry fibroblasts (patient 125), which took up the secreted enzyme. The cells were separated by a 0.4-μm pore size membrane, cocultivated for 48 hr, and analyzed for enzyme activity. #125/TF1 NI, Fabry fibroblasts cocultivated with nontransduced TFI cells; #125/TF1 I, Fabry fibroblasts cocultivated with transduced TFI cells; #125/TFI I/M-6-P, Fabry fibroblasts cocultivated with transduced TF1 cells in the presence of 5 m*M* mannose 6-phosphate; #125/CD34 NI, Fabry fibroblasts cocultivated with nontransduced CD34+ cells; #125/CD34 I, Fabry fibroblasts cocultivated with transduced CD34+ cells; #125/CD34 I/M-6-P, Fabry fibroblasts cocultivated with transduced CD34+ cells in the presence of 5 m*M* mannose 6-phosphate.

demonstrated that the secreted enzyme from human CD34+ cells transduced with MFG-α-gal A corrected the enzyme deficiency in Fabry fibroblasts. Transduced CD34+ cells can be used as an enzyme source to correct neighboring or distant enzyme-deficient Fabry fibroblasts. This strongly suggests the possibility of gene therapy targeted to hematopoietic stem/progenitor cells for the treatment of Fabry disease, even though hematopoietic cells are not a primary part of the disorder.

After transduction, the enzyme activity is much higher in TFI cells than in CD34+ cells. The reasons for this may include a higher transduction efficiency for the virus in TFI cells than in CD34+ cells, and differences in vector expression within transduced cells. To increase the transduction efficiency of CD34+

cells, we used centrifugal enhancement, which we have previously shown to increase the transduction of several kinds of cells including $CD34^+$ cells (Bahnson *et al.*, 1995; Takiyama *et al.*, 1998).

By this technique we obtained a high transduction efficiency of 63% (42–88%) for $CD34^+$ cells. Centrifugation is a simple and safe technique and can be applied in clinical studies (Nimgaonkar *et al.*, 1995).

The amount of α-gal A enzyme activity required for the reversal of phenotype of this disease is not known. Studies in the glucuronidase-deficient model of mucopolysaccharidosis VII demonstrated that a small correction of the enzymatic deficiency (about 5% of normal) resulted in the reversal of organomegaly and other signs of the disease (Wolfe *et al.*, 1992). This correction was achieved by transplantation of transduced bone marrow cells. In the current study, expression of α-gal A is severalfold above normal. We also showed that expressing α-gal A in transduced $CD34^+$ cells resulted in its secretion. It is likely that secretion of α-gal A could contribute to a therapeutic effect in patients transplanted with genetically corrected hematopoietic stem/progenitor cells.

We have begun clinical trials of gene therapy in Gaucher disease. In this protocol $CD34^+$ cells were obtained from patient blood by leukapheresis. After mobilization with granulocyte colony stimulation factor (G-CSF), white blood cells were collected by leukapheresis and a density gradient separation of peripheral blood mononuclear cells (PBMCs) was performed. The PBMCs were enriched for $CD34^+$ cells by immunoaffinity column purification and transduced *ex vivo* with supernatant from the amphotropic producer cells. The genetically corrected cells were then transplanted back to the patients without ablation (Barranger *et al.*, 1995). The same strategy can be applied for clinical trials of gene therapy for Fabry disease.

ACKNOWLEDGMENTS

We thank Dr. Paul Robbins for providing, the MFG plasmid, and Drs. Hideaki Tahara and Yasuhiko Nishioka for helpful suggestions. This work was supported by the National Gaucher Foundation, the Metropolitan Government, and the Ministry of Education, Science and Culture of Japan.

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Received for publication September 2, 1998; accepted after revision September 14, 1999.

QH301.F4

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CODEN: HGTHE3; ISSN: 1043-0342

882

Isolation and Characterization of the mouse Phosphoenolpyruvate Carboxykinase (muPEPCK) Gene. C.P. Williams, C.Postic, M.A. Magnuson, and R. Chalkley. Vanderbilt Univ., Nashville, TN 37232

A bacterial artificial chromosome library, derived from mouse genomic DNA (strain 129 SV), was screened with a mouse PEPCK cDNA fragment. One positive clone, ~25 kbp, was isolated. Sequence from this clone was identical to the mouse cDNA, confirming that the locus isolated contains the mouse PEPCK gene. Comparison of muPEPCK to the rat gene shows a high degree of homology within the exons and at the exon/intron boundaries. The transcription start site was identified by primer extension and was shown to be conserved with the rat PEPCK mRNA start site. Northern blot analysis of total RNA isolated from mouse liver shows that the mRNA is 2800 nt in length, similar to that seen in rat. Additionally, RNA isolated from the livers of mice fasted overnight shows higher levels of PEPCK mRNA than from mice fed ad lib, which is in accordance with the regulation observed for rat PEPCK mRNA. Currently, we are examining whether regulatory regions identified as important for the expression of the rat PEPCK gene are conserved in the mouse gene. Sequence comparison of the proximal promoter indicates ~90% conservation. Furthermore, several hypersensitive (HS) sites previously identified in our laboratory in H4IIE cells, a rat hepatoma cell line, also appear to be conserved in mouse liver. HS sites have been shown to correlate with protein binding in regions important for gene expression. This suggests that important upstream regulatory regions, as well as the proteins that bind to them, are conserved amongst these species.

GENE TRANSFER AND THERAPY (883-886)

883

TETRACYCLINE - CONTROLLED GENE EXPRESSION SYSTEM ACHIEVES BOTH HIGH LEVEL AND QUANTITATIVE CONTROL OF GENE EXPRESSION. Dong X. Yin and Li Zhu. CLONTECH Laboratories, Inc., 1020 E. Meadow Circle, Palo Alto, CA 94303.

The tetracycline-controlled gene expression system utilizes the control elements of the tetracycline-resistance operon encoded in Tn10 of *E. coli* to control gene expression in eukaryotic cells. Here we demonstrate the quantitative control of the expression of luciferase gene, dihydrofolate reductase gene, and bcl-2 gene in HeLa S3 or Chinese hamster ovary AA8 cells using the tetracycline-controlled gene expression system. Regardless of the host cell lines or the genes being expressed, there is a common range of tetracycline concentration within the expression of genes in most sensitively regulated. In addition, the maximal gene expression level of the tetracycline-controlled gene expression systems is higher than that of the wild-type CMV promotor/enhancer-driven systems. Nonetheless, careful section of stably transfected clones is necessary to achieve the optimally regulated gene expression using this system.

884

MODULATION OF GENE EXPRESSION BY ANTISENSE OLIGONULCLEOTIDES AND EXPRESSION PLASMIDS TRANSFECTED WITH STARBURST™ PAMAM DENDRIMERS. Anna Bielinska, Jolanta F. Kukowska-Latallo, Jennifer Johnson, Donald A. Tomalia, and James R. Baker, Jr. University of Michigan, Ann Arbor, MI 48109 and Michigan Molecular Institute, Midland, MI48640.

Starburst polyamidoamine (PAMAM) dendrimers are a new synthetic polymer characterized by a branched spherical shape and a high density surface charge. We have investigated the ability of these dendrimers to function as an effective delivery system for antisense oligonucleotides and "antisense expression plasmids" for the targeted modulation of gene expression. Dendrimers bind to various forms of nucleic acids on the basis of electrostatic interactions, and the ability of DNA/dendrimer complexes to transfer oligonucleotides and plasmid DNA to mediate antisense inhibition was assessed in *in vitro* cell culture system. Cell lines that permanently express luciferase gene were developed using dendrimer mediated transfection. Transfections of antisense oligonuc-leotides or antisense cDNA plasmids using dendrimers resulted in a specific and dose dependent inhibition of luciferase expression. This inhibition caused approximately 25 - 50% reduction of baseline luciferase activity, depending on the oligonucleotide or antisense plasmid DNA concentrations, generation of the dendrimer and charge ratio of the DNA/dendrimer complexes. Binding of the oligonucleotide to the dendrimer extended intracellular survival of the unmodified DNA. Complexing with dendrimers does not change sequence specificity of the antisense oligonucleotides or RNA. Our results indicate that Starburst dendrimers can be effective carriers for the introduction of regulatory nucleic acids and facilitate the suppression of the specific gene expression.

885

GENE THERAPY FOR FABRY DISEASE: EXPRESSION, SECRETION AND UPTAKE OF α-GALACTOSIDASE A (α-gal A) DRIVEN BY A HIGH TITER RECOMBINANT RETROVIRAL VECTOR. J. A. Medin, M. Tudor, R. Simovitch, J. M. Quirk, S. Jacobson, G. J. Murray, and R. O. Brady. Unit on Therapeutic Gene Transfer, Develop. and Met. Neurol. Branch, Neuroimmun. Branch, NINDS, NIH, Bethesda, MD 20892.

Fabry disease is an X-linked metabolic disorder due to an enzymatic deficiency of α-gal A. Affected males accumulate glycosphingolipids with terminal α–gal residues in many tissues and succumb to renal or vascular disease. No specific therapy exists. Towards correction, a retrovirus has been constructed that efficiently expresses the α-gal A gene. A high titer producer line was found to express large amounts of intracellular and also secreted α-gal A. Fabry patient's skin fibroblasts and a patient B cell line were corrected by infection and enzyme secretion was observed. Secreted enzyme was taken up by uncorrected patient's cells in a man-6-P receptor-dependent manner thus also providing therapeutic activity in *trans*. Further studies will examine the transduction efficiency and enzyme production in normal and patients' CD34+-enriched hematopoietic stem/progenitor cells towards the goal of genetic therapy for this disorder.

886

Enhanced tumor recognition and killing using the HSV-TK suicide gene. Ramesh R. Freeman SM, Munshi A, Abboud CN, Marrogi AJ. Department of Pathology, Tulane Medical Center, New Orleans.

Genetically altered tumor cells expressing the Herpes Simplex Virus thymidine kinase gene (HSV-TK) is being used in the treatment of cancers based on the ability of the HSV-TK gene modified cells to kill nearby unmodified cells, termed as the "bystander effect". Using this system, we have demonstrated in an *i.p.* therapeutic murine tumor model that the "bystander effect" was generated by a cytokine cascade post inoculation of HSV-TK gene modified tumor cells. TNF, IL-1, IL-6, IL-2, IFN-γ and GM-CSF could be detected within the tumor by RT-PCR at various times post injection. Semi quantitative PCR showed an increase in TNF-α mRNA with time after treatment. We observed a hemorrhagic tumor necrosis and could demonstrate the influx of T cells and macrophages within the tumor post treatment. Based on these observations we proposed that the HSV-TK gene modified cells can alter the tumor microenvironment from inhibitory to one that is stimulatory. The present study investigated whether HSV-TK treatment could induce the expression of the costimulatory molecules ICAM, B7-1 and B7-2 on lymphocytic infiltrates in vivo. In vivo, analysis for B7-1, B7-2 and ICAM expression by immunohistochemistry and RT-PCR revealed significant expression ($p=0.031$) on tumor infiltrating mononuclear cells from mice receiving HSV-TK treatment in comparison to mice not receiving the treatment. Furthermore, tumor infiltrating lymphocytes isolated from tumors after inoculation of the HSV-TK gene modified cells showed a significant increase in proliferation in response to syngeneic tumor cells. The results of this study suggest that the HSV-TK suicide gene can stimulate the release of cytokines within the tumor which induces the expression of costimulatory molecules and subsequent stimulation of tumor infiltrating lymphocytes. (Supported by CAGNO grant to AJM).

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2108

Towards Development of a Gene Therapy f r Fabry dis ase. *N.S.Yew*[1], *R. Ziegler*[1], *P. Berthelette*[1], *J. Marshall*[1], *M. Cherry*[1], *C. Li*[1], *H. Romanczuk*[1], *R.J. Desnick*[2], *Y.A. Ioann u*[2], *and S.H. Cheng*[1]. [1]Genzyme Corp., 1 Mountain Rd., Framingham, MA 01701 and [2]Mount Sinai Sch. of Med., New York, NY 10029.

Fabry Disease is a recessive, X-linked disorder caused by deficient alpha-galactosidase A activity, leading to a progressive deposition of the glycosphingolipid globotriaosylceramide (GL3) in most tissues of the body. We are developing a gene therapy to treat this disease using both viral and non-viral approaches, and have constructed a recombinant adenoviral vector (pAd2-hAGA) and a plasmid expression vector (pCFA-hAGA) that expresses human alpha-galactosidase A. Fibroblasts from a Fabry patient that were either infected or transfected with these vectors expressed active enzyme at levels significantly higher than endogenous levels in normal fibroblasts. A portion of the expressed enzyme was secreted and shown to be taken up by untransfected fibroblasts. Initial studies have begun in mice using plasmids expressing the chloramphenicol acetyltransferase (CAT) reporter gene to compare the efficacy of three potential routes of delivery - intranasal, intravenous, and intramuscular administration. Intranasal instillation of the reporter plasmid pCF1-CAT complexed with the cationic lipid GL-67 resulted in high levels of CAT expression (up to 400 ng CAT per 100 mg tissue) in the lung. Intravenous administration of pCF1-CAT complexed with another cationic lipid, GL-89, also resulted in CAT activity primarily in the lung with lesser amounts in the heart, spleen, kidney and liver. Intramuscular injection of pCF1-CAT DNA in the absence of cationic lipid produced high levels of CAT expression (>300 ng per 100 mg tissue) in the injected muscle. Preliminary experiments performed in alpha-galactosidase deficient Fabry mice indicate that low levels of enzyme activity can be detected in several organs after administration of either naked DNA or lipid:DNA complex by these different delivery methods. Current efforts are directed to find the optimal delivery vehicle and route of administration, and to determine if this therapy can reduce the levels of GL3 in the Fabry mouse.

RB155-8-1-185

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Correction of Enzymatic and Lysosomal Storage Defects in Fabry Mice by Adenovirus-Mediated Gene Transfer

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ABSTRACT

Fabry disease is a recessive, X-linked disorder caused by a deficiency of the lysosomal hydrolase α-galactosidase A. Deficiency of this enzyme results in progressive deposition of the glycosphingolipid globotriaosylceramide (GL-3) in the vascular lysosomes, with resultant distension of the organelle. The demonstration of a secretory pathway for lysosomal enzymes and their subsequent recapture by distant cells through the mannose 6-phosphate receptor pathway has provided a rationale for somatic gene therapy of lysosomal storage disorders. Toward this end, recombinant adenoviral vectors encoding human α-galactosidase A (Ad2/CEHα-Gal, Ad2/CMVHIα-Gal) were constructed and injected intravenously into Fabry knockout mice. Administration of Ad2/CEHα-Gal to the Fabry mice resulted in an elevation of α-galactosidase A activity in all tissues, including the liver, lung, kidney, heart, spleen, and muscle, to levels above those observed in normal animals. However, enzymatic expression declined rapidly such that by 12 weeks, only 10% of the activity observed on day 3 remained. α-Galactosidase A detected in the plasma of injected animals was in a form that was internalized by Fabry fibroblasts grown in culture. Such internalization occurred via the mannose 6-phosphate receptors. Importantly, concomitant with the increase in enzyme activity was a significant reduction in GL-3 content in all tissues to near normal levels for up to 6 months posttreatment. However, as expression of α-galactosidase A declined, low levels of GL-3 reaccumulated in some of the tissues at 6 months. For protracted treatment, we showed that readministration of recombinant adenovirus vectors could be facilitated by transient immunosuppression using a monoclonal antibody against CD40 ligand (MR1). Together, these data demonstrate that the defects in α-galactosidase A activity and lysosomal storage of GL-3 in Fabry mice can be corrected by adenovirus-mediated gene transfer. This suggests that gene replacement therapy represents a viable approach for the treatment of Fabry disease and potentially other lysosomal storage disorders.

OVERVIEW SUMMARY

In this study, we evaluated the ability of adenovirus-mediated gene transfer of α-galactosidase A to correct the enzymatic and storage defects in a murine model of Fabry disease. We showed that restoration of normal enzymatic activity and reduction of GL-3 storage in the visceral organs could be attained after a single intravenous administration of the recombinant adenovirus vector. However, enzyme expression was transient, with resultant reaccumulation of GL-3. To address this limitation, we showed that repeated administration of the recombinant adenovirus vector could be facilitated by use of an antibody against the CD40 ligand. Together, these data support the use of gene replacement strategies for the treatment of Fabry disease.

INTRODUCTION

LYSOSOMAL STORAGE DISORDERS are caused by an inherited deficiency of one or more of the several lysosomal enzymes that normally catalyze the metabolism of glycoproteins, glycolipids, and other macromolecules (Neufeld, 1991; Gieselmann, 1995). Fabry disease, an example of such a storage dis-

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ease, is an X-linked, recessive disorder resulting from a deficiency in α-galactosidase A (Brady *et al.*, 1967). Absence of this lysosomal glycosidase leads to the accumulation of high levels of incompletely metabolized glycosphingolipids, principally globotriaosylceramide (GL-3), that have terminal α-galactosyl residues (Desnick *et al.*, 1995). Progressive deposition of these glycosphingolipids in the vascular endothelium and visceral tissues of patients leads to the development of angiokeratoma, hypohydrosis, corneal and lenticular opacities, episodic pain crises in the extremities, and severe renal impairment. Death in early adulthood usually occurs from renal failure or from cardiac or cerebrovascular disease.

The genomic sequences and cDNAs encoding both the human and mouse α-galactosidase A have been isolated (Bishop *et al.*, 1986, 1988; Ohshima *et al.*, 1995; Gotlib *et al.*, 1996). With the availability of the murine gene, a mouse model of Fabry disease has been generated by targeted disruption of the α-galactosidase A gene locus (Wang *et al.*, 1996; Ohshima *et al.*, 1997). These α-galactosidase A-deficient mice appear clinically normal but display accumulation of GL-3 in several organs, similar to that observed in Fabry patients. The accumulation of GL-3 in these mice progresses with age, with a substantial increase in concentration between 2 and 5 months and a stabilization at approximately 6 months. This similarity in metabolite accumulation in the Fabry knockout mice and in subjects with Fabry disease, coupled with the slow progression of the disease process in the animals, makes this model suitable for evaluation of various therapeutic strategies.

A proportion (5 to 20%) of several of the hydrolases implicated in lysosomal storage disorders normally are secreted rather than trafficked directly to lysosomes (Von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). However, these secreted enzymes can be recaptured by adjacent and distant cells via the mannose 6-phosphate receptor. Other receptor-mediated pathways may also be involved (Pfeffer, 1991). This observation of a mechanism for secretion and recapture of lysosomal enzymes has provided a therapeutic rationale for this group of disorders based on either enzyme replacement or somatic gene therapy. In the case of Gaucher disease, treatment of patients (type I) with purified enzyme (β-glucocerebrosidase) has been shown to be effective, and to result in clinical improvement (Pastores *et al.*, 1993). On the basis of these observations, methods to produce and purify quantities of recombinant α-galactosidase A have also been developed (Ioannou *et al.*, 1992), and their use in Fabry patients is currently being evaluated. However, these enzymes have short half-lives in circulation and are rapidly cleared, thereby requiring frequent readministrations with relatively large quantities of the enzyme. This requirement for frequent repeated infusions with large boluses of enzyme increases the likelihood of generating an immune response to the recombinant enzyme, particularly in those patients who harbor null mutations. Such a host immune response may preclude subsequent treatments with the enzyme.

An alternative approach that addresses some of these limitations is to modify a population of cells genetically *in vivo* such that continuous and prolonged secretion of the enzyme into the systemic circulation could be attained. Several different gene delivery vector systems that may be suitable in this regard are currently under investigation (Poenaru, 1996). *In vitro* studies using these gene transfer vctors have indicated that they are capable of correcting the enzyme deficiency in affected cells (Akli *et al.*, 1996; Medin *et al.*, 1996; Di Francesco *et al.*, 1997), and *in vivo* studies using animal models have shown partial restoration of enzyme activity and reduction of storage products (Moullier *et al.*, 1993; Salvetti *et al.*, 1995; Ohashi *et al.*, 1997). Prompted by these observations, we evaluated the feasibility of gene therapy using a recombinant adenovirus vector encoding α-galactosidase A to correct both the enzyme and storage defects in the Fabry mouse model.

MATERIALS AND METHODS

Recombinant adenovirus vectors

A previral plasmid was constructed that contained adenovirus type 2 (Ad2) sequences flanking an expression cassette composed of a cytomegalovirus (CMV) enhancer-E1a promoter, a hybrid intron, the human α-galactosidase A cDNA, and the simian virus 40 (SV40) polyadenylation signal. The Ad2/CEHα-Gal virus was generated by homologous recombination of the previral plasmid with Ad2/βGal-4 (Armentano *et al.*, 1995; Jiang *et al.*, 1996) followed by plaque purification. Ad2/CMVHIα-Gal is similar to Ad2/CEHα-Gal, except that it contains both the CMV enhancer and promoter sequences. Both recombinant adenovirus vectors are deleted of the E1 region but retain E3 and E4. Early studies were performed using Ad2/CEHα-Gal. In the interim, it was shown that greater persistence of expression in the lung could be attained with adenoviral vectors that harbor a complete E4 region in conjunction with the CMV promoter (Armentano *et al.*, 1997). In consequence some of the later studies were performed using Ad2/CMVHIα-Gal. Ad2/CFTR-16 encodes the cystic fibrosis transmembrane conductance regulator and was constructed as described previously (Scaria *et al.*, 1998). Methods for producing and purifying the viruses were as described by Armentano *et al.* (1995). The ratios of particles to infectious units were typically between 1:10 and 1:100.

Cell lines

The human Fabry fibroblast cell line (Repository No. GM02775) was obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ). Cells were maintained in Eagle's minimal essential medium (MEM)–Earle's balanced salt solution (BSS) containing a 2× concentration of essential and nonessential amino acids, vitamins, and 20% (v/v) fetal bovine serum at 37°C and 5% CO_2.

Animals

Four- to 6-week-old female BALB/c, 129/Sv, and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Fabry (–/–) mice (Wang *et al.*, 1996) were bred at Genzyme (Framingham, MA) and allowed to mature to at least 4 months of age before use. Preliminary studies have shown that there were no differences in α-galactosidase A levels or in the rate of accumulation of GL-3 between female and male Fabry (–/–) mice. For most of the studies described here, only 4- to 6-month-old female Fabry (–/–) animals were used.

Mice were anesthetized by metaphane inhalation and injected through the tail vein with either 250 μl of the recombinant adenovirus vectors or phosphate-buffered saline (PBS). The animals were perfused with PBS just prior to removing the organs, which were then quick-frozen on dry ice and stored at −80°C until ready for processing. Blood was also collected from the obital venous plexus under anesthesia using heparinized microhematocrit capillary tubes at various times postinjection.

Measurement of α-galactosidase A

Tissues were weighed, homogenized in lysis buffer (27 m*M* citric acid, 46 m*M* sodium phosphate dibasic, 1% Triton X-100, and 1× protease inhibitor cocktail [Boehringer Mannheim, Indianapolis, IN], pH 4.6), and adjusted to a final concentration of 250 mg of tissue per milliliter of lysis buffer. The homogenized samples were subjected to three rapid freeze–thaw cycles and then stored at −80°C. For analysis, the frozen homogenates were first thawed and clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. The presence of α-galactosidase A in the supernatants was determined using either 5 m*M* 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal) in the presence of 117 m*M* *N*-acetylgalactosamine (inhibitor of α-galactosidase B) at pH 4.4 as described previously (Ioannou *et al.*, 1992), or by using an enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody to α-galactosidase A. This polyclonal antibody was generated in rabbits by using highly purified recombinant human α-galactosidase A isolated from Chinese hamster ovary cells. Characterization of this polyclonal antibody showed that it recognized the human α-galactosidase A with greater affinity than it did murine α-galactosidase A. In studies in which α-galactosidase A was measured using 4MU-α-Gal, conversion was performed using a specific activity of 30 U/mg for the enzyme.

For the ELISA, 96-well microtiter plates (Corning, Corning, NY) were first coated with the rabbit anti-α-galactosidase serum by incubating the plates with a solution containing antibody (1.5 μg/ml) in 0.1 *M* $NaHCO_3$, pH 9.5, for 1 hr at 37°C. The plates were then blocked with 5% nonfat dry milk (Bio Rad, Hercules, CA) in TBST (0.05 *M* Tris-HCl, 0.1 *M* NaCl, 0.05% Tween 20, pH 7.5) at 4°C for a minimum of 1 hr and then washed three times with ELISA plate wash buffer (300 μl/well; NEN Life Sciences, Boston, MA) using a model 1575 Immunowash plate washer (Bio-Rad). Samples diluted in 5% nonfat dry milk in TBST were loaded onto the plates and incubated for 1 hr at 37°C. The plates were washed a further six times with ELISA plate wash buffer and then incubated with biotinylated anti-α-galactosidase serum (1.25 μg/ml) (biotinylation was accomplished using the EZ-Link-Sulfo-NHS-LC biotinylation kit from Pierce, Rockford, IL) at 37°C for 1 hr. After six additional rinses with ELISA plate wash buffer, the plates were incubated with 1 μg/ml streptavidin–horseradish peroxidase (HRP) (1 μg/ml; Pierce) at 37°C for 30 min. The plates were subjected to six additional washes with ELISA plate wash buffer and then developed by incubating with a solution containing a 100-mg/ml concentration of 3,3′,5,5′-tetramethyl benzidine dihydrochloride in substrate buffer (240 m*M* citric acid, 520 m*M* Na_2HPO_4, pH 5.0) in a darkened room at room temperature for up to 30 min. The reactions were stopped by adding 100 μl of 2 *M* H_2SO_4 to each well and the absorbance intensities at 450 nm were determined using a Bio-Rad model 450 plate reader. Concentrations were calculated from a standard curve generated using purified recombinant human α-galactosidase A (50 to 1000 pg/ml).

Uptake of α-galactosidase A by Fabry fibroblasts

Plasma was collected from Fabry mice 3 days after administration of 1×10^{11} particles of Ad2/CEHα-Gal. The plasma (containing approximately 1 μg of α-galactosidase A per milliliter) was mixed with growth medium containing either 5 m*M* mannose 6-phosphate or mannose 1-phosphate and then added to the Fabry fibroblast cell line GM02775. These cells were normally incubated with the respective sugars for 1 hr prior to addition of the plasma. After an additional incubation for 24 hr at 37°C, the cells were washed extensively with PBS and then lysed by addition of 0.25 ml of lysis buffer. The amounts of intracellular α-galactosidase A were quantitated using the 4MU-α-Gal assay.

Quantitation of GL-3 levels

GL-3 quantitation was carried out as described (Zeidner *et al.*, 1999). Briefly, tissues were homogenized in chloroform–methanol (2:1, v/v) at a ratio of 0.1 ml/mg wet tissue. Plasma (30 μl) was extracted with 0.6 ml of chloroform–methanol (2:1, v/v) by vortexing. After a 15-min incubation at 37°C on a rocking platform, samples were centrifuged to remove cell debris and a one-fifth volume of water was added to the equivalent of 5 mg of tissue or to 25 μl of plasma. The phases were allowed to separate at 4°C for 24 hr and then centrifuged to complete the separation. The lower phase (chloroform) was transferred to a clean glass tube and dried under nitrogen. To purify glycosphingolipids, the dried lipids were resuspended in 1 ml of chloroform and 0.5 mg equivalent applied to 500-mg Lichrolut RP-18 columns (EM Sciences, Gibbstown, NJ). After washing with chloroform, the neutral glycosphingolipids were eluted from the columns with acetone–methanol (9:1, v/v), dried under nitrogen, and then resuspended in ethanol. Quantitation of GL-3 in the samples was performed using an ELISA that relies on the affinity of the glycolipid for the *Escherichia coli* verotoxin B subunit (VTB). The lipids in ethanol (equivalent to 12.5 to 100 μg of tissue or 2.5 μl of plasma) were applied to 96-well PolySorp plates (VWR Scientific Products, Bridgeport, NJ) and dried to completion by incubation at 37°C. After blocking with 5% bovine serum albumin in Tris-buffered saline (TBS) for 1 hr at 37°C, the wells were reacted sequentially with VTB (400 ng/well), a monoclonal antibody against VTB (1 μg/well), and alkaline phosphatase-conjugated goat anti-mouse IgG. Wells were developed with *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine, pH 9.6, at room temperature. The reactions were stopped with 100 μl of 5% EDTA and read at 405 nm in a Bio-Rad 450 plate reader. Standard curves were generated with porcine GL-3 (Matreya, Pleasant Gap, PA) using 5 to 100 ng/well.

In situ *localization of α-galactosidase A in tissue sections*

Naive or adenovirus-treated Fabry mice were euthanized with sodium pentobarbital, then perfused with PBS followed by

2% paraformaldehyde. After 10 min, the organs were dissected, rinsed in assay buffer (27 m*M* citric acid and 46 m*M* dibasic sodium phosphate, pH 4.4), cut into 1- to 5-μm sections, and then placed in staining solution (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside, 0.5 mg/ml in assay buffer containing 7 m*M* potassium ferricyanide and 7 m*M* potassium ferrocyanide) for 2 to 4 hr at room temperature. After staining, the sections were rinsed with PBS and counterstained with eosin.

Histopathology

Tissues were fixed at 30 cm of water pressure with 2% paraformaldehyde and 0.2% glutaraldehyde. Representative samples were embedded in glycol methacrylate, sectioned, and stained with hematoxylin and eosin. Histopathology was evaluated in a blinded fashion and graded subjectively using a scale of 0 to 4, where a score of 0 indicates no abnormal findings and a score of 4 reflects severe changes (Scheule *et al.*, 1997). Serum samples were also collected and analyzed for evidence of hepatic injury.

Transient immunosuppression

Transient immunosuppression of mice was attained using MR1 (Noelle *et al.*, 1992), a purified hamster anti-mouse CD40 ligand monoclonal antibody (PharMingen, San Diego, CA). Mice were injected intraperitoneally with a total of six injections of 500 μg of MR1/mouse, starting on day -1 relative to the time of administration of the adenovirus vector. Subsequent injections of MR1 were on days 1, 4, 7, 10, and 14 postadministration of the virus. For the repeat administration studies, animals were administered first with Ad2/CFTR-16, an adenovirus vector encoding the cystic fibrosis transmembrane conductance regulator (Scaria *et al.*, 1998), and then challenged 28 days later with Ad2/CEHα-Gal.

RESULTS

Levels of α-galactosidase A and GL-3 in normal and Fabry mouse tissues

A mouse model for Fabry disease has been generated by targeted disruption of the murine α-galactosidase A gene (Wang *et al.*, 1996). To test whether this animal model displayed characteristics similar to those observed in Fabry patients, transgenic Fabry (–/–) mice (4–6 months of age) and age-matched normal C57BL/6 mice were sacrificed and the levels of α-galactosidase A and GL-3 in various tissues were determined. Figure 1A shows that the Fabry mice, unlike the C57BL/6 animals, exhibited only low levels of α-galactosidase A activity in all of the tissues assayed. The residual enzyme activity detected in the Fabry tissues was likely attributable to α-galactosidase B, which also exhibits some ability to hydrolyze the 4MU-α-Gal substrate used in this assay, despite the presence of inhibitor (Mayes *et al.*, 1981). β-Hexosaminidase activity remained unchanged in the tissues of the mutant mice (data not shown).

The levels of the glycosphingolipid GL-3 in Fabry and wild-type mice were determined using an ELISA (Fig. 1B). All tissues from the wild-type mice contained essentially undetectable levels of GL-3 whereas tissues from Fabry animals revealed significant accumulation of GL-3. Highest levels of storage were observed in the kidney and spleen, with lowest levels in the heart. This is somewhat different from Fabry patients, who invariably display highest levels of glycosphingolipid accumulation in the kidney, followed by the heart, liver, and spleen. Elevated levels of GL-3 were also detected in the plasma of the Fabry mice (data not shown). Together, these data indicate that the transgenic Fabry mice are deficient in α-galactosidase A activity with consequent accumulation of high levels of GL-3 in all organs.

Restoration of α-galactosidase A activity in Fabry mouse tissues after administration of Ad2/CEHα-Gal

In an attempt to correct the enzyme deficiency in the Fabry mice, animals were injected via the tail vein with 1.6×10^{11} particles of Ad2/CEHα-Gal, a recombinant adenovirus vector encoding human α-galactosidase A. The animals were sacrificed after 3 days and the level of α-galactosidase A in various organs determined using an ELISA. A low level of cross-reactive material (signal was marginally above background) was detected in the untreated animals (Fig. 2). In contrast, after intravenous injection of Ad2/CEHα-Gal, high levels of α-galactosidase A were detected in the liver, with more than 50 μg of α-galactosidase A/100 mg of tissue, and from 1 to 10 μg of α-galactosidase A/100 mg of tissue detected in the heart, spleen, kidney, lung, and right quadriceps (Fig. 2). These levels were several logs higher than those observed in untreated Fabry mice and were 10- to 100-fold greater than those observed in wild-type C57BL/6 mice. Quantities of the enzyme (5 to 10 μg/ml) were also detected in the plasma on day 3. Using the ELISA, the levels of α-galactosidase A in wild-type C57BL/6 mice were not detectable. We also assayed and detected small amounts of α-galactosidase A in brain homogenates (data not shown), which was likely due to the presence of the enzyme in the vasculature.

In situ histochemical staining of tissue sections revealed that more than 70% of the hepatocytes of treated animals were positive for α-galactosidase A, with lower levels in cells of the spleen and kidney (Fig. 3). In the liver, staining was distributed evenly throughout the organ whereas in the spleen, staining was observed primarily in the red pulp and excluded from the white pulp. Within the kidney, staining was observed in the outer cortex, where it was confined mainly to the glomeruli; there was also scattered staining in the inner portion of the medulla. No staining was detected in untreated Fabry or in wild-type C57BL/6 mice (Fig. 3). The absence of a signal even in the wild-type C57BL/6 mice is consistent with the quantitative assays described above, demonstrating that Ad2/CEHα-Gal-infected animals harbored levels of α-galactosidase A that were 1 to 2 logs greater than those observed in normal animals. These results suggest that restoration of α-galactosidase A levels to greater than normal could be attained in the Fabry mice after a single administration of Ad2/CEHα-Gal.

Uptake of plasma-derived α-galactosidase A by Fabry fibroblasts

The preceding results did not distinguish whether the high levels of α-galactosidase A expression measured in the different organs were a direct result of infection of the cells by the

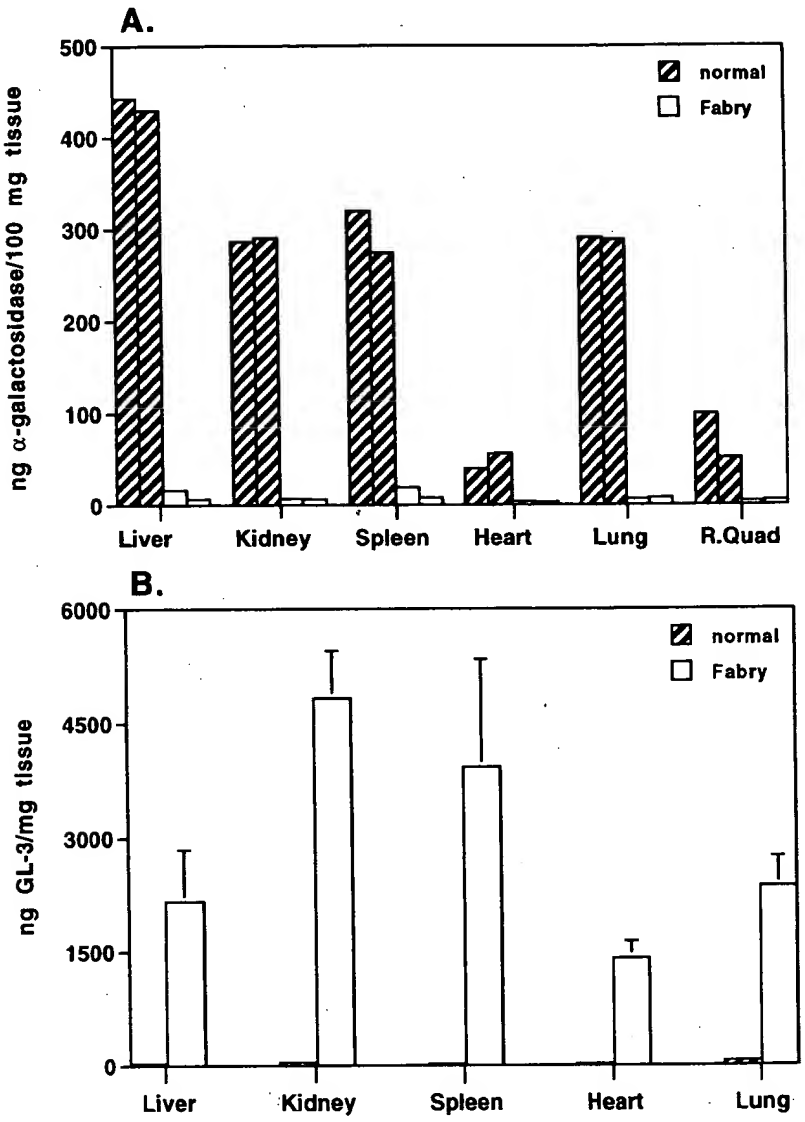

FIG. 1. Levels of α-galactosidase A and GL-3 in normal and Fabry mice tissues. Age-matched Fabry and C57BL/6 mice were sacrificed and the levels of **(A)** α-galactosidase A and **(B)** GL-3 in the liver, kidney, spleen, heart, lung, and right quadriceps were quantitated. Enzyme levels were determined using the fluorescent substrate 4-methylumbelliferyl-α-D-galactopyranoside and GL-3 levels were assayed using an ELISA as described in Materials and Methods. The levels of α-galactosidase A shown were from individual animals (two each of Fabry and C57BL/6). GL-3 levels were an average from four animals. Data are expressed as means ± SEM.

adenovirus vector, or rather a result of cells taking up enzyme that had been secreted into the systemic circulation. To determine if cells could take up the enzyme produced from infected cells and secreted into the blood, Fabry mice were injected intravenously with 1×10^{11} particles of Ad2/CEHα-Gal. Blood was collected 3 days postinjection and the plasma added to the culture medium of human Fabry fibroblasts (GM02775) to a final concentration of 1 μg of α-galactosidase A per milliliter. The cells were incubated overnight at either 37 or 4°C, then washed extensively and assayed for intracellular α-galactosidase A activity. Measurable levels of enzyme were present in cells exposed to plasma from the adenovirus-treated mice (Fig. 4). This uptake of α-galactosidase A was effectively blocked when the cells were preincubated with mannose 6-phosphate but not mannose 1-phosphate, indicating that internalization of the hydrolase occurred via a mannose 6-phosphate receptor-mediated pathway (Fig. 4). The uptake was also inhibited when the cells were cultured at 4°C, implying that metabolic activity was required for internalization of the enzyme (Fig. 4). These results demonstrate that α-galactosidase A present in the circulation of adenovirus-treated Fabry mice was in a form that could be internalized by Fabry fibroblasts. This suggests that at least some of the α-galactosidase A detected in the organs of Ad2/CEHα-Gal-infected Fabry mice could have been due to uptake of secreted enzyme from the systemic circulation.

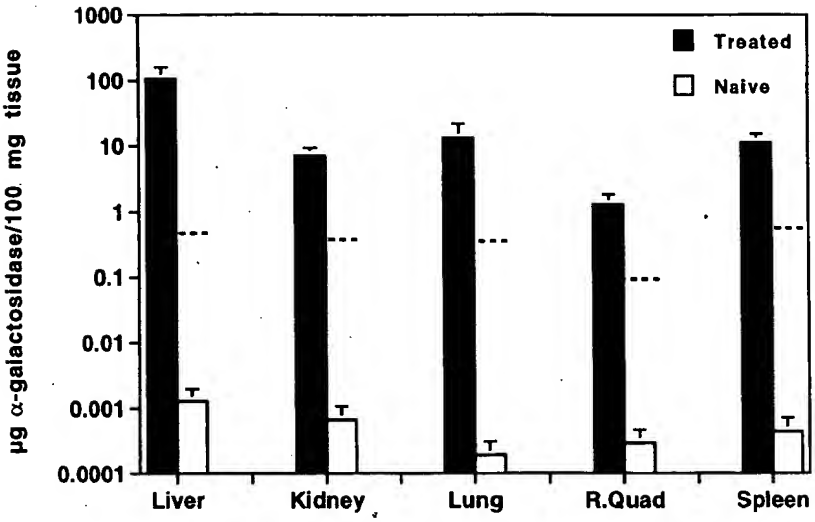

FIG. 2. Effect of intravenous administration of Ad2/CEHα-Gal to Fabry mice. Fabry mice were injected through the tail vein with 1.6×10^{11} particles of Ad2/CEHα-Gal (treated) or were untreated (naive). Three days postinjection, the animals were sacrificed and the levels of α-galactosidase A in the tissues were measured using an ELISA as described. The dashed lines indicate the approximate level of α-galactosidase A determined in normal C57BL/6 mouse tissues. Data are expressed as means ± SEM ($n = 4$).

Dose optimization of Ad2/CEHα-Gal for expression of α-galactosidase A

Given the high levels of expression of α-galactosidase A attained with 1.6×10^{11} particles of Ad2/CEHα-Gal, we sought to determine the minimum dose sufficient to ameliorate the enzyme and storage defects in the Fabry animals. We also elected to use Ad2/CMVHIα-Gal, which harbors the CMV promoter and enhancer, in the hope that greater persistence of transgene expression could be realized compared with the CMV enhancer-E1a hybrid promoter in Ad2/CEHα-Gal. Using BALB/c mice as surrogates, animals were injected via the peripheral vein with 1×10^{11}, 5×10^{10}, 1×10^{10}, or 5×10^{9} particles of Ad2/CMVα-Gal and the levels of α-galactosidase A in the liver, kidney, and spleen assayed. As was observed with Ad2/CEHα-Gal, high levels of the hydrolase were detected in all of these organs on day 3 postinfection with 10^{11} particles of Ad2/CMVα-Gal (Fig. 5). Surprisingly, treating the mice with a 2-fold lower vector dose resulted in at least a 10- to 100-fold decrease in α-galactosidase A levels. At a dose of 1×10^{10} particles, levels in the liver decreased nearly 1000-fold, with similar decreases in the spleen and kidney (Fig. 5). This nonlinear dose response was also observed in the plasma (data not shown). At the lowest dose of 5×10^{9} particles, low levels of α-galactosidase A were detected in the liver and spleen, but near background levels were found in the kidney and plasma. Hence, it would appear that a threshold dose of adenovirus was required to see significant levels of α-galactosidase A in the tissues and plasma; above this threshold, increasing doses resulted in highly nonlinear increases in expression.

Time course of α-galactosidase A expression after administration of Ad2/CMVHIα-Gal

To determine the duration of α-galactosidase A expression in the different organs, 1×10^{11} particles of Ad2/CMVα-Gal were injected into the tail veins of Fabry mice. The mice used for this experiment had an average age of 5 ± 1 month. Blood was taken by eye bleed at several time points and organs were harvested at 1, 4, 12, and 24 weeks postinjection. The organs were divided into halves and assayed for α-galactosidase A and GL-3 levels. An ELISA was used to measure the levels of α-galactosidase A in tissue homogenates and plasma samples. The highest levels of α-galactosidase A were detected in the liver, with lower levels in the kidney, lung, spleen, and heart (Fig. 6A). Expression in each of these organs decreased 5- to 10-fold from 1 to 4 weeks postinjection, and an additional 10-fold from 4 to 12 weeks postinjection. However, it should be noted that despite this rate of decrease, the expression levels of α-galactosidase A at 12 weeks were still at or above the α-galactosidase A levels present in normal untreated C57BL/6 mice (Fig. 6A). Expression in all of the organs was essentially undetectable by 24 weeks postinjection. The levels of α-galactosidase A in the plasma (Fig. 6B) followed a time course similar to that seen in the tissues. High levels of enzyme were detected in the circulation on day 3 postinjection (approximately 5 μg/ml plasma) but the level declined steadily over time, such that only background levels were detected after 16 weeks. This transience of expression was likely due to the immune response to the adenovirus and transgene product as evidenced by the presence of antibodies to both adenovirus and human α-galactosidase A in the plasma (data not shown). It should be noted that because the knockout Fabry mice lack α-galactosidase A, the human α-galactosidase A likely acts as a neoantigen in these animals.

Levels of GL-3 in tissues from Fabry mice treated with Ad2/CMVHIα-Gal

Tissues of Fabry mice treated with Ad2/CMVHIα-Gal as described above were also analyzed for their content of GL-3.

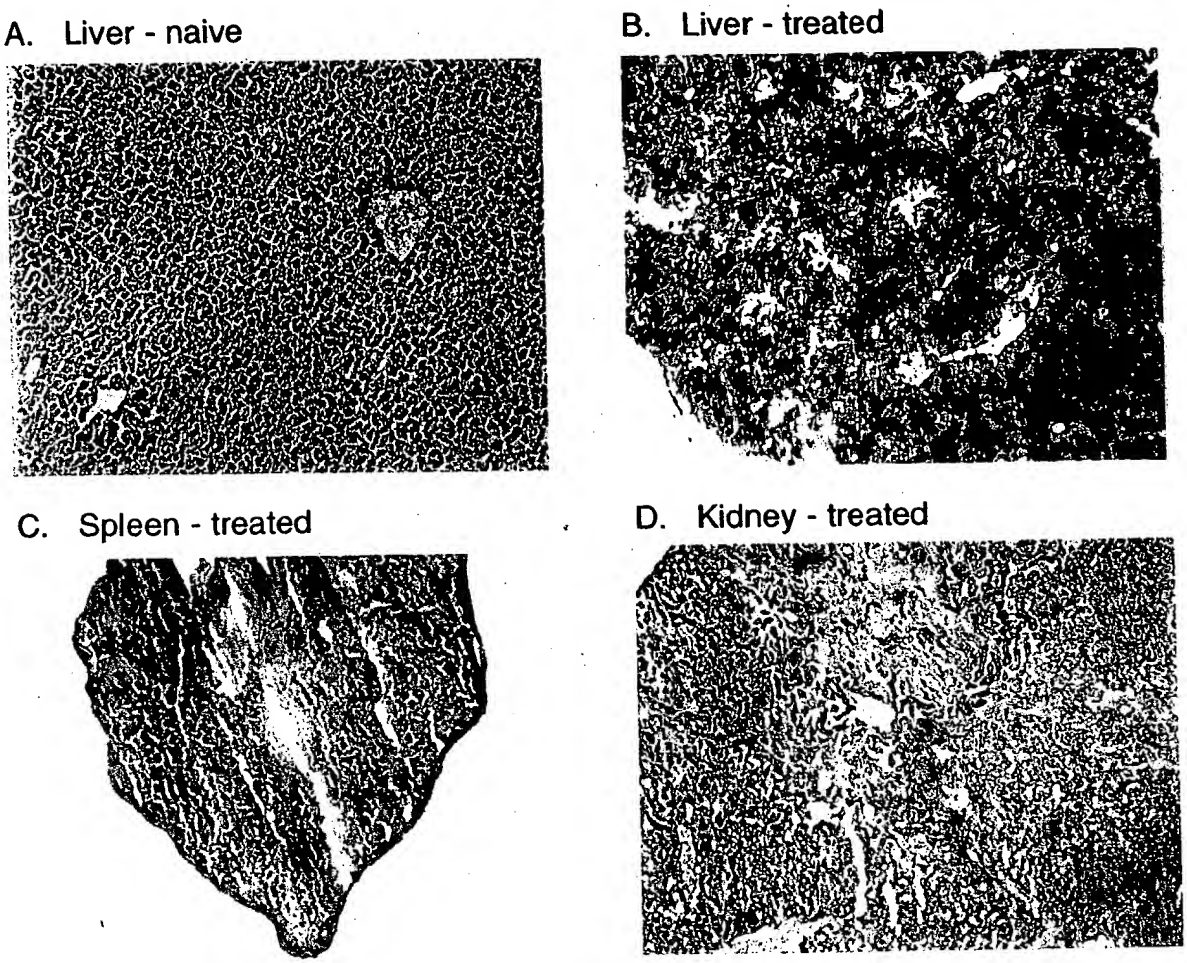

FIG. 3. Histochemical staining of tissues of Fabry mice after administration of Ad2/CEHα-Gal. Fabry mice were injected through the peripheral vein with 1.65×10^{11} particles of Ad2/CEHα-Gal and tissues were harvested 3 days postinjection. Frozen sections were stained using 5-bromo-4-chloro-3-indoly-α-D-galactopyranoside, an α-galactosidase substrate. Shown are **(A)** untreated liver, **(B)** treated liver, **(C)** treated spleen, and **(D)** treated kidney at an original magnification of ×40.

With the demonstration that therapeutic levels of α-galactosidase A could be attained over a period of 12 weeks (Fig. 6), we sought to determine the kinetics of clearance and reaccumulation of GL-3 over this time. The levels of GL-3 in the tissues of Ad2/CMVHIα-Gal-treated Fabry mice were compared with those in untreated age-matched animals. Figure 7 shows that with the exception of the kidney, GL-3 levels in the liver, spleen, heart, and lung were decreased to near background levels by 1 week postinjection. Clearance of GL-3 was least effective in the kidney, with reduction to only approximately 20% of untreated controls at 1 week. They remained at these low levels for up to 12 weeks posttreatment but began to reaccumulate low amounts of the glycosphingolipid by 24 weeks. At the 24-week time point, the levels of GL-3 in the liver, heart, and lung were still lower than in untreated age-matched animals but those in the spleen and kidney had increased to 30 to 60% of the levels observed in Fabry animals (Fig. 7). This profile of clearance and reaccumulation of GL-3 in the various organs was consistent with the observed expression profile of α-galactosidase A. As the levels of the enzyme in the circulation declined after 12 weeks, GL-3 levels in the tissues reaccumulated such that by 24 weeks postinfection, significant levels of GL-3 were observed, particularly in the kidney and spleen. These observations indicate that the abnormal storage of GL-3 in the Fabry mice could be reduced by a single administration of Ad2/CMVHIα-Gal for up to 6 months posttreatment.

Toxicity associated with administration of Ad2/CEHα-Gal

To assess the safety of administration of recombinant adenovirus vectors, BALB/c mice were injected via the peripheral vein with 5×10^{10}, 1×10^{11}, or 5×10^{11} particles of Ad2/CEHα-Gal and sacrificed on days 3, 7, and 14 postinjection for analysis. Animals that received the highest dose exhibited significant weight loss that was most severe on day 7 postinfection but that returned to near normal limits by day 14 (Fig. 8A). As reported previously, toxicity was largely confined to the liver (Yang *et al.*, 1994; Lieber *et al.*, 1996), and was

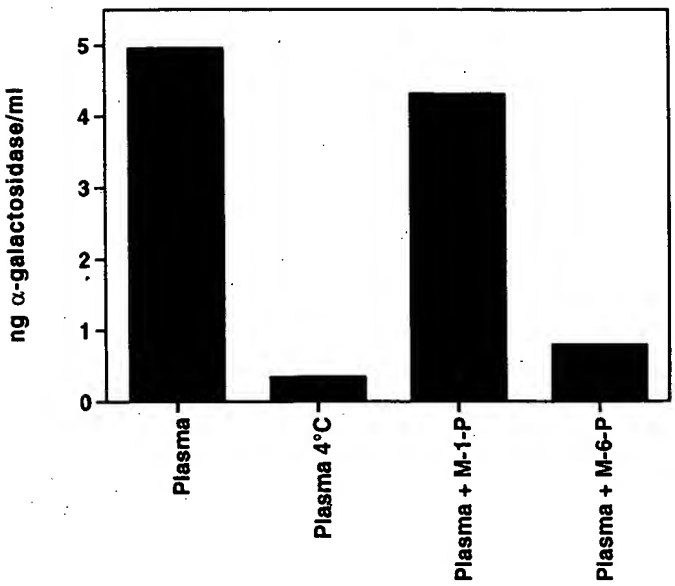

FIG. 4. Uptake of α-galactosidase A from plasma of Fabry mice treated with Ad2/CEHα-Gal. Fabry mice were injected via the tail vein with 1×10^{11} particles of Ad2/CEHα-Gal. Plasma was collected 3 days postinjection and then added to the culture medium of the Fabry fibroblast cell line GM02775 to a final concentration of approximately 1 μg of α-galactosidase A per milliliter. The cells were pretreated for 1 hr with either mannose 1-phosphate (M-1-P) or mannose 6-phosphate (M-6-P) or at 4°C. The media containing plasma-derived α-galactosidase A (and containing either M-1-P or M-6-P) were then added to the respective group of cells and incubated for a further 24 hr at 37°C. Cells were washed extensively with PBS and the levels of intracellular α-galactosidase A determined in the cell lysate, using the fluorescent substrate 4-methylumbelliferyl-α-D-galactopyranoside. Results shown are an average of two separate studies.

characterized by hepatocellular hypertrophy, vacuolation and necrosis, and the presence of mononuclear and mixed cell infiltrates (Fig. 8B). Pathological lesions were also noted in the spleen and were characterized most prominently by extramedullary hematopoiesis and lymphoid hyperplasia (Fig. 8C). No significant findings were observed in either the lung, kidney, heart, or ovary. Dose-dependent elevation of serum transaminases (ALT, AST) and alkaline phosphatase levels

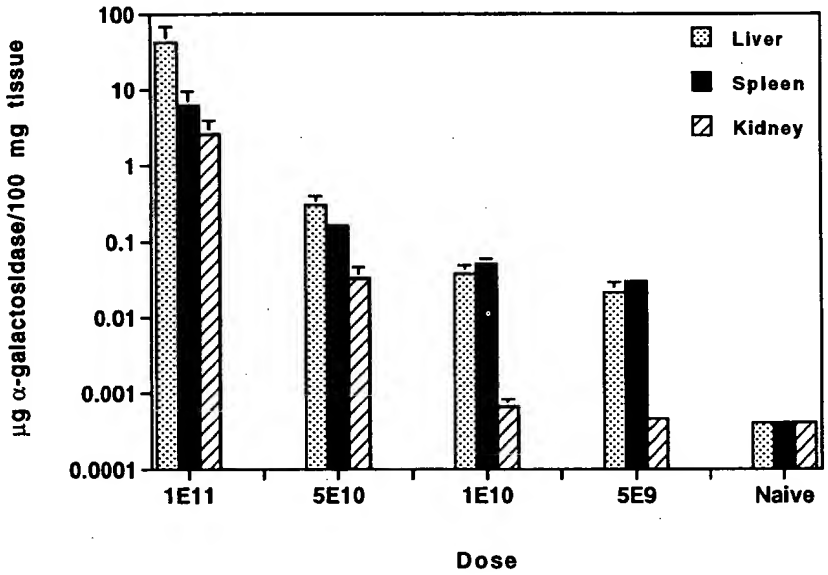

FIG. 5. Expression of α-galactosidase A in the tissues of BALB/c mice injected with increasing doses of Ad2/CMVHIα-Gal. BALB/c mice were injected via the tail vein with between 5×10^9 and 1×10^{11} particles of Ad2/CMVHIα-Gal. The animals were sacrificed 3 days postinjection and an ELISA was used to measure the levels of α-galactosidase A in the liver, spleen, and kidney. Naive animals were untreated. Data are expressed as means ± SEM ($n = 5$).

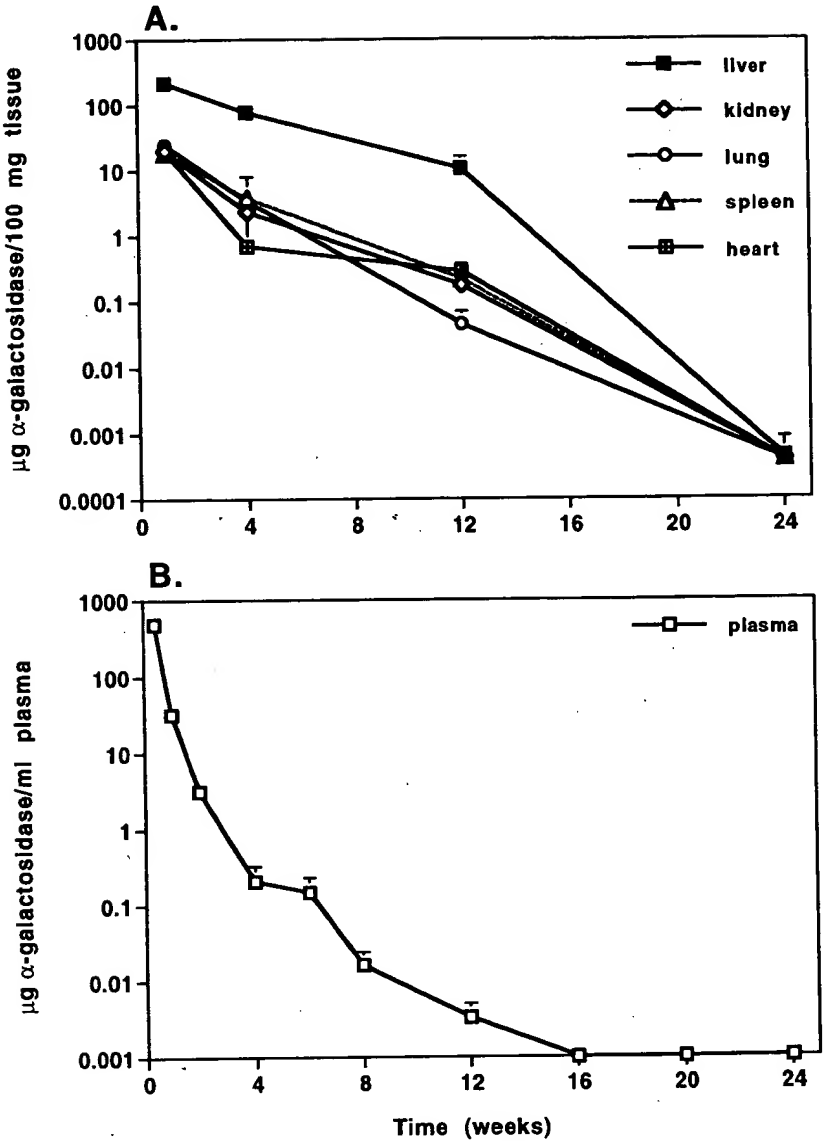

FIG. 6. Persistence of α-galactosidase A expression after intravenous administration of Ad2/CMVHIα-Gal. Age-matched Fabry mice were injected via the tail vein with 1×10^{11} particles of Ad2/CMVHIα-Gal. **(A)** Groups of four animals were sacrificed at weeks 1, 4, 12, and 24 postinjection and their organs assayed for α-galactosidase A activity. **(B)** Blood was collected on day 2, and on week 1, 2, 4, 6, 8, 12, 16, 20, and 24 postinjection and the levels of α-galactosidase A in the plasma were determined using an ELISA. Data are presented as means ± SEM ($n = 4$).

were detected as well as a dose-dependent reduction in blood glucose levels (Fig. 9).

Efficacy of repeated administration of adenovirus vectors, using transient immunosuppression

As transgene expression was found to be transient (Fig. 6), we tested the feasibility of repeat dosing with adenovirus vectors. A broad range of immunosuppressants has been tested for their ability to abate the immune response to adenoviral vectors. These include, for example, the use of agents that are capable of disrupting either the CD40–CD40 ligand or B7–CD28 interactions (Kay *et al.*, 1995, 1997; Yang *et al.*, 1996; Scaria *et al.*, 1997; Stein *et al.*, 1998). Previous studies have indicated that transient inhibition of both costimulatory molecule interactions may be required for efficient readministration of adenovirus to the liver. In this study, we evaluated whether treatment with an antibody to CD40 ligand (MR1) alone, and over a shorter time than had been previously reported, might suffice to allow an effective secondary adenovirus administration.

BALB/c mice were given a primary infusion (1×10^{11} particles) of recombinant adenovirus encoding CFTR (Ad2/CFTR-16), followed by a secondary infusion of Ad2/CEHα-Gal on day 28. The animals were sacrificed 3 days after the second injection and the levels of α-galactosidase A in the liver, spleen, and kidney were assayed by an ELISA. Mice that had received

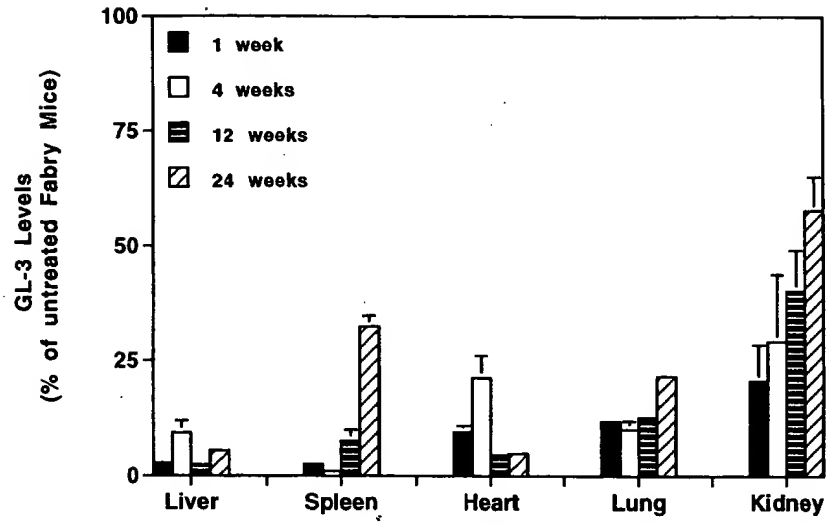

FIG. 7. Levels of GL-3 in tissues of Fabry mice administered Ad2/CMVHIα-Gal. Organs from the mice injected with 1×10^{11} particles of Ad2/CMVHIα-Gal as outlined in the caption to Fig. 6 were processed and assayed for the levels of GL-3, using an ELISA. Shown are levels of GL-3 in the liver, spleen, heart, lung, and kidney at weeks 1, 4, 12, and 24 postinjection. The levels of GL-3 are expressed as a percentage of the levels found in age-matched untreated Fabry mice. Data are presented as means ± SEM ($n = 4$).

MR1 (500 μg/mouse on days −1, 1, 4, 7, 10, and 14 relative to the first injection) expressed high levels of α-galactosidase A after the second injection, indicating that efficient secondary gene transduction occurred (Fig. 10). The levels achieved were comparable to those observed in naive animals that received a single administration of Ad2/CEHα-Gal. In contrast, only low levels of α-galactosidase A were detected in animals administered a second dose of adenovirus in the absence of MR1 treatment (Fig. 10). These data indicate that effective readministration of adenovirus is possible if the initial dose is accompanied by a transient blockade of T cell costimulation.

DISCUSSION

Several factors suggest that gene transfer may represent an attractive modality to treat lysosomal storage disorders such as Fabry disease. First, a proportion of the lysosomal hydrolases that are normally secreted into systemic circulation is capable of being recaptured by distant cells through specific cell receptors (Neufeld, 1991). This indicates that genetic modification of an appropriate depot organ such as the liver, lung, or muscle may suffice to produce corrective levels of the deficient enzyme to reverse the diseased state in the visceral organs (Ohashi *et al.*, 1997; Vogler *et al.*, 1998). Second, only low and unregulated levels of the affected enzymes need to be restored for therapeutic correction (Wolfe *et al.*, 1992; Moullier *et al.*, 1993; Desnick *et al.*, 1995). Heterozygotes with as little as 10% of normal enzyme activity are otherwise healthy. This observation argues that high-efficiency gene transduction may not be necessary for therapeutic efficacy. Third, enzyme replacement therapy has been shown to result in clinical improvements in both animal models and patients (Barton *et al.*, 1991; Pastores *et al.*, 1993; Shull *et al.*, 1994; Crawley *et al.*, 1996; O'Connor *et al.*, 1998). On the basis of these observations, we examined whether recombinant adenovirus vectors encoding human α-galactosidase A could facilitate correction of the enzymatic and lysosomal storage defects in a murine model of Fabry disease.

Restoration of enzymatic activity and reduction of lysosomal storage in Fabry mice could be attained after a single administration of Ad2/CEHα-Gal

The availability of the Fabry mouse model facilitates the evaluation of different therapeutic approaches for this disease. Several modalities are currently being evaluated, including enzyme and gene replacement, and pharmacologic therapies (Desnick *et al.*, 1995; Medin *et al.*, 1996; Platt *et al.*, 1997). Here, we show that restoration of α-galactosidase A activity to normal levels and reduction of GL-3 to basal levels could be attained in most Fabry tissues after a single infusion of a recombinant adenovirus vector. Quantities of α-galactosidase A that were 10- to 100-fold higher than normal levels in a broad array of organs were attained. Although these levels declined over time, they remained above wild-type levels over a period of 4 weeks. With the exception of the kidney, accumulated GL-3 was cleared as early as 1 week posttransduction and the levels of GL-3 remained low for up to 3 months. Some reaccumulation of the glycosphingolipid was observed in two of the organs (spleen and kidney) after 6 months, consistent with the loss of expression of α-galactosidase A. These observations are also concordant with those showing that significant accumulation in newborn Fabry mice occurs only after 3 to 5 months, with the highest levels being observed in the spleen and kidney.

The tissue distribution of enzyme activity that we observed was consistent with that obtained by adenoviral vectors that express reporter genes (Huard *et al.*, 1995; Peeters *et al.*, 1996). Hence it is possible that the activity in the various organs was due to uptake of the recombinant adenoviral vector rather than

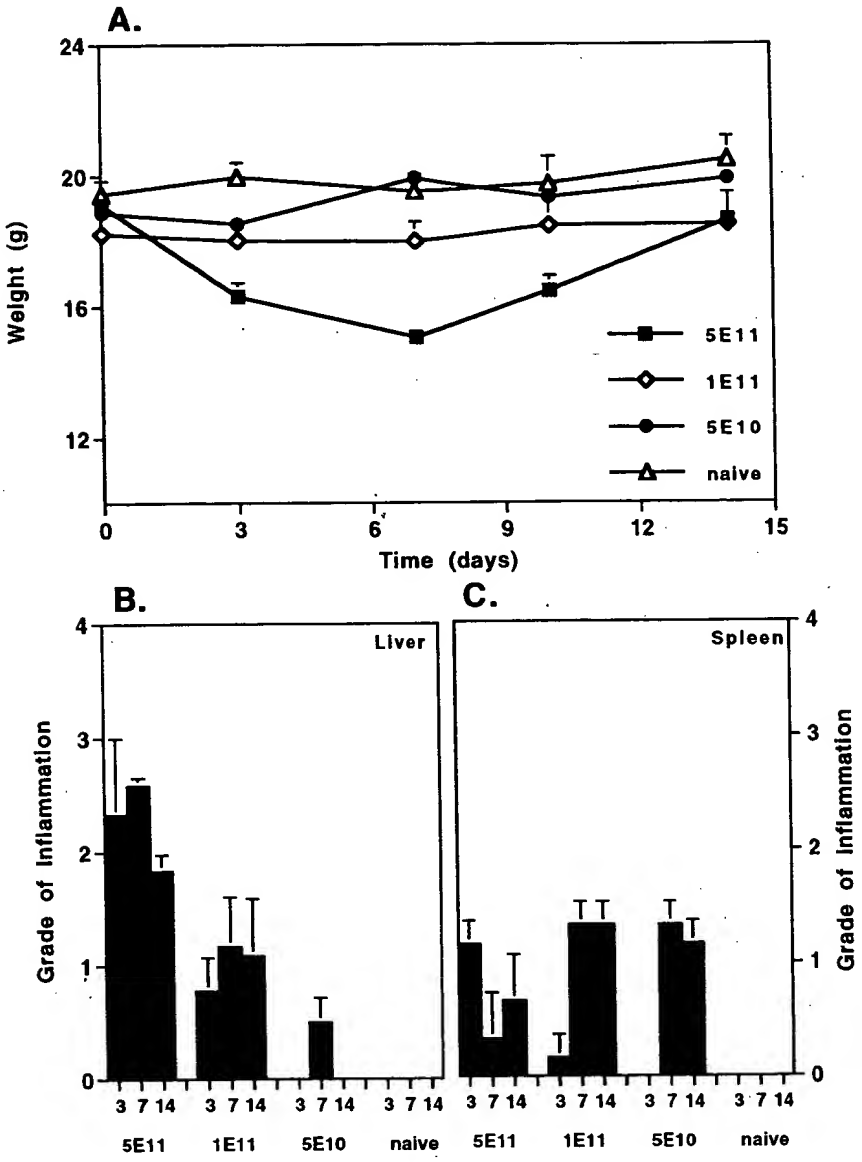

FIG. 8. Toxicity associated with intravenous administration of increasing doses of Ad2/CEHα-Gal. BALB/c mice were administered between 5×10^{10} and 5×10^{11} particles of Ad2/CEHα-Gal via the tail vein. **(A)** The weights of the animals were monitored twice weekly over a 2-week period. **(B)** Groups of mice were also sacrificed on days 3, 7, and 14 postinjection and their organs processed for histopathological examination in a blinded manner. Inflammation was graded on a scale of 0 to 4, with 0 indicating no change, and 4 indicating a severe change from normal. Of the organs analyzed (liver, lung, spleen, kidney, heart, ovaries), only changes in the **(B)** liver and **(C)** spleen were observed and are shown here. Naive animals were administered with PBS. Data are presented as means ± SEM ($n = 4$).

to the uptake of the secreted, recombinant enzyme. However, high levels of circulating α-galactosidase A in a form that was competent for internalization by the mannose 6-phosphate receptor were generated from the adenoviral expression vector. This would suggest that at least some of the α-galactosidase A activity found in the organs was likely due to uptake of the secreted enzyme. The observation of near complete elimination of detectable GL-3 in the liver, spleen, heart, and lung at the early time points also supports this suggestion. It is unlikely that infection of these organs alone could account for the observed high degree of GL-3 depletion. Furthermore, other lysosomal enzymes secreted from genetically modified cells have also been shown to be capable of being internalized by cells of different organs (Moullier *et al.*, 1993, 1995; Ohashi *et al.*, 1997; Tsujino *et al.*, 1998; Watson *et al.*, 1998).

Potential limiting issues

Despite the observed efficacy of this approach, there are limitations associated with the use of adenoviral gene transfer vectors. First, we and others have observed a dose-dependent toxicity, particularly hepatic injury, subsequent to the intravenous administration of the recombinant adenovirus vector. Although these inflammatory events tended to resolve with time, some

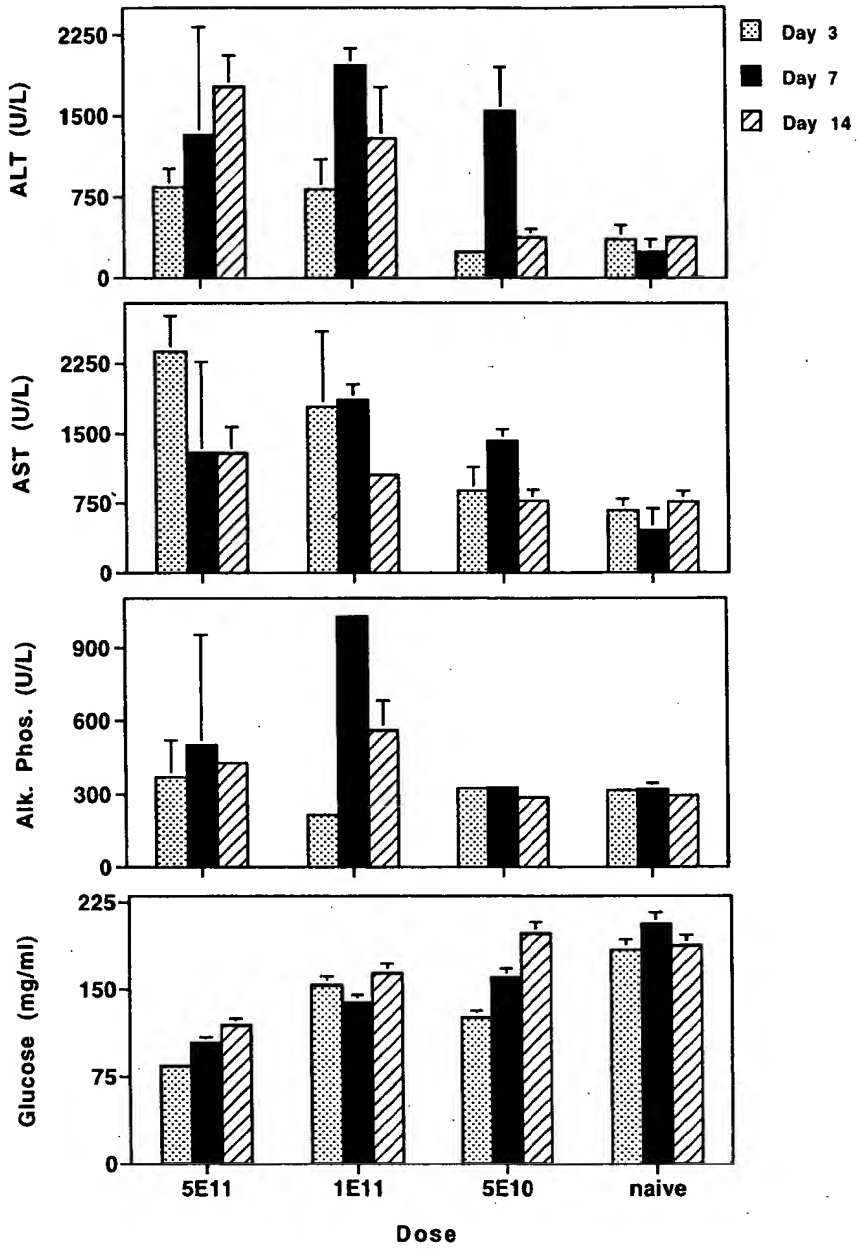

FIG. 9. Changes in serum chemistry after administration of Ad2/CEHα-Gal. Blood was collected from BALB/c mice treated as indicated in the caption to Fig. 8 on days 3, 7, and 14 postinjection with Ad2/CEHα-Gal. Levels of serum transaminases (ALT, AST), alkaline phosphatase (alk. phos.) and glucose in treated animals as well as those observed in normal (naive) animals are indicated. Data are presented as means ± SEM ($n = 4$).

consideration to minimize these inflammatory responses is clearly desirable. In this regard, it has been reported that removal or inactivation of additional viral genes, such as E2a and E4, from the viral backbone generated vectors with an improved safety profile (Engelhardt *et al.*, 1994; Gao *et al.*, 1996). Complete removal of all adenoviral coding sequences also offers the prospect of decreased cellular toxicity in response to the viral proteins (Schiedner *et al.*, 1998). Alternatively, because recombinant adeno-associated viral vectors are essentially devoid of viral genes, they represent yet another possible gene transfer system for use in this disease indication (Xiao *et al.*, 1998; Snyder *et al.*, 1999). The ability to reduce vector-associated toxicity is an important consideration not only because it improves the safety profile of these delivery systems but it also resulted in greater persistence of transgene expression (Connelly *et al.*, 1996; Gao *et al.*, 1996; Dedieu *et al.*, 1997; Morral *et al.*, 1997).

Given that greater than normal levels of α-galactosidase A resulted from 10^{11} particles of Ad2/CEHα-Gal, a possible approach to reduce the extent of vector-induced hepatic toxicity was to reduce the dose. However, *in vivo* dose optimization studies revealed a nonlinear dose response. A 2-fold reduction in adenoviral particles was accompanied by a greater than 100-

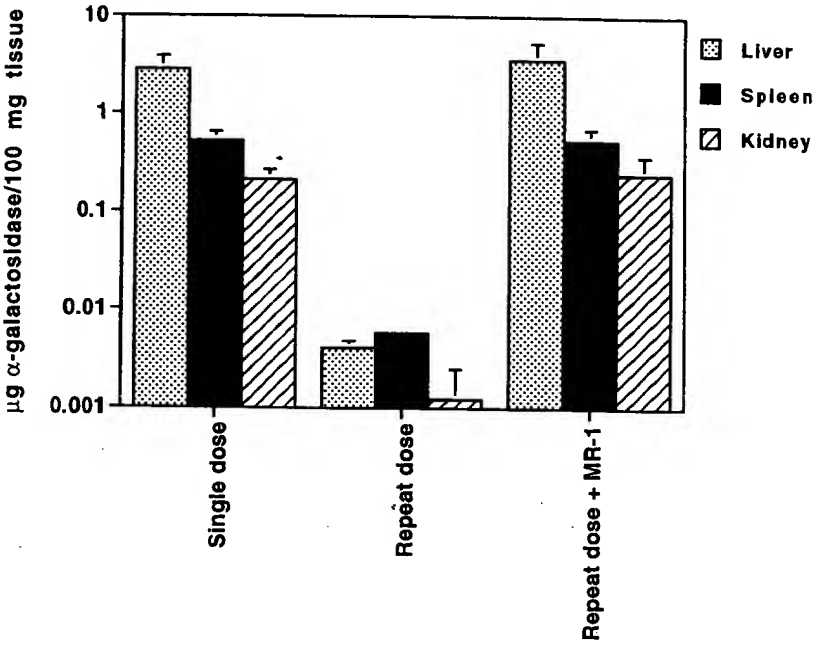

FIG. 10. Efficacy of readministration with recombinant adenovirus vectors. Two groups of four BALB/c mice each were injected via the tail vein with 1×10^{11} particles of Ad2/CFTR-16. One group (Repeat dose + MR-1) was injected intraperitoneally with 500 μg of the anti-CD40 ligand antibody MR1 on days -1, 1, 4, 7, 10, and 14 relative to virus administration while the other (Repeat dose) was untreated. Twenty-eight days after the first virus administration, the mice were injected with 1×10^{11} particles of Ad2/CEHα-Gal. A third group of mice (Single dose) received only Ad2/CEHα-Gal on day 28. Three days after the second injection, all of the animals were sacrificed and organs harvested. Tissue homogenates were then assayed for α-galactosidase A expression using an ELISA. Data are presented as means $\pm$ SEM ($n = 4$).

fold decrease in enzyme expression in some of the tissues. A similar observation has been reported using an adenoviral vector encoding factor VIII (Connelly *et al.*, 1996). The basis for this lack of linearity in dose response is unclear, but may perhaps be related to the extent of inflammation in the liver and its possible influence on gene expression. Alternatively, some proportion of the virus may be sequestered after systemic administration, thereby requiring a threshold quantity before high levels of gene transduction can be attained. Further studies are warranted to understand this nonlinear dose response.

Furthermore, although Ad2/CEHα-Gal mediated high levels of α-galactosidase A in both tissues and plasma, the clinical utility of this vector system may be limited by the attenuation of expression at 12 weeks. Only approximately 10% of the peak activity remained at 12 weeks, and this declined to residual levels by 24 weeks. An accumulating body of data suggests that this loss in expression is due, at least in part, to an immunologic response to component(s) of the adenoviral vector and the transgene product (Engelhardt *et al.*, 1994; Yang *et al.*, 1994; Dai *et al.*, 1995; Tripathy *et al.*, 1996; Michou *et al.*, 1997). Antibodies to adenovirus and α-galactosidase A were detected as early as 1 week postadministration, which likely led to the elimination of transduced cells and removal of circulating enzyme. Other factors may also be involved in extinguishing expression including loss of vector and promoter shutoff. Presently, several strategies have been shown to be effective in minimizing these effects. These include, among others, use of immunosuppressants (Kay *et al.*, 1997; Jooss *et al.*, 1998), tolerization with adenoviral proteins (Ilan *et al.*, 1996; DeMatteo *et al.*, 1997), and vector modification (Engelhardt *et al.*, 1994; Armentano *et al.*, 1997; Bruder *et al.*, 1997; Schiedner *et al.*, 1998).

Yet another impediment to using an adenoviral delivery system is the inability to readminister the adenovirus vector. However, it is becoming apparent that transient immunosuppression using a variety of chemicals (cyclophosphamide, FK506, or deoxyspergualin), soluble CTLA4Ig, and antibody to CD40 ligand is effective in overcoming this barrier (Jooss *et al.*, 1996; Smith *et al.*, 1996; Yang *et al.*, 1996; Kaplan and Smith, 1997; Kay *et al.*, 1997; Scaria *et al.*, 1997; Stein *et al.*, 1998). We confirm here that by using the monoclonal antibody MR1 to block the costimulation between T cells and antigen-presenting cells and B cells, high levels of transgene expression could be attained after a second intravenous administration of adenovirus. Administration of MR1 inhibited the development of neutralizing antibodies and decreased the cellular immune response to the adenoviral vector (Yang *et al.*, 1996; Scaria *et al.*, 1997). However, although only transient blockade of T cell costimulation with MR1 was sufficient to allow readministration of virus, the need to coadminister recombinant antibodies to patients adds to the complexity of the treatment.

In summary, we have shown that intravenous administration of a recombinant adenoviral vector can mediate the expression of α-galactosidase A levels, sufficient to reverse the accumulation of glycosphingolipids in the visceral organs of the Fabry mouse for up to 6 months. It is clear that with the development

of improved viral vectors that are less immunogenic, more conducive to repeated administration, and that provide greater longevity of transgene expression, gene therapy will be useful for treatment of Fabry disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the Virus Production Group, histology and LARD staff for technical assistance, and Dave Garlick for histopathology analysis. We also thank members of the Gene Transfer Research Group, as well as Samuel Wadsworth, Judith St. George, Richard Gregory, Ronald Scheule, Donna Armentano, Abraham Scaria, Debra Barngrover, and Geoffrey Akita, for helpful discussions.

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Received for publication February 5, 1999; accepted after revision April 19, 1999.